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From the Department of Zoofysiology University of Uppsala, Sweden

The Occurrence of ß-Carotene in Blood Cells from Cattle

By

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Abstract

Nilsson, B *The occurrence of β carotene in blood cells from cattle* Acta physiol scand 1971 83 1—3

Blood cells were prepared from cattle sucking calves and pigs and the chief part of the hemoglobin was removed by hemolysis The remains were disintegrated with ultrasonic vibration and extracted with phosphate buffer after which they were freeze-dried and finally extracted with iso-octane Spectrophotometrical registrations of the iso-octane extracts obtained from blood cells of adult cattle showed that the extracs contained β-carotene The concentration of β-carotene in the disintegrated blood cells was estimated at about 0 5 mg % In cattle β-carotene thus occurs not only in the blood plasma as stated in the literaure but also in the blood cells The iso-octane extracts obtained from blood cells of sucking calves or pigs contained no measurable quantities of β-carotene on account of which the β-carotene in the blood cells from adult cattle seems to derive from the fodder

In isolating a special substance from blood cells from cattle the presence of a carotenoid in the cells was regularly observed The aim of this investigation was to identify this carotenoid material and to shed light on any possible connection with the fodder supplied

The basic material used was blood from cattle sucking calves and pigs Freshly heparinized blood was obtained from the Uppsala branch of the Stockholm Gavle Meat marketing Association 1 ml of heparin solution (1000 IE/ml) in 20 ml saline was added to each litre of blood

Methods

The blood cells in 480 ml of blood were centrifuged in a MSE High speed 18 centrifuge at 20 000 × *g* for 20 min and the plasma was drawn off The volume of the sedimented blood cells was about 250 ml In order to remove the chief part of the hemoglobin the erythrocytes were then hemolysed by the adddition of 4 times the volume of a 0 015 *M* HCl solution which made the pH about 5 75 If not the pH was adjusted to this value The centrifugation of the erythrocyte ghosts was facilitated by the dilution owing to the reduction of the high viscosity of the suspension The sedimentation was also facilitated by the fact that the ghosts aggregated on account of the low pH value which is close to their iso-electric point The ghosts were centrifuged at 20 000 × *g* for 1 hr The supernatant was carefully drawn off and discarded The pellet of erythrocyte ghosts which prob[illegible]

fragment of them was suspended in twice its volume of a 0 005 M phosphate buffer (pH 7 0) The suspension was subjected to ultrasonic vibration (MSE 100 watt ultrasonic disintegrator) in small portions at 16 kc/s for 1 min and left to extract for about 24 hrs at +2° C The material treated in this way was centrifuged at 20 000 g for 1 hr and the supernatant was drawn off The carotenoid material was then isolated from the disintegrated blood cells which had been extracted with phosphate buffer and which weighed 15 g It was freeze dried and then pulverized in a porcelain mortar and extracted with iso-octane in a Soxhlet apparatus In the process the flask containing the extraction preparation was kept submerged in a water bath at a thermostat controlled temperature of +30° C The cooling fluid which was taken from a freezer had a temperature of −30° C and the pressure in the apparatus was about 35 mm Hg The extraction lasted for 6 hrs The extraction capsules in the apparatus had been extracted free of impurities for a day before they were used

The iso-octane used was "spectrographically pure (Phillips Petroleum Company) The other chemicals were of the purest qualities that could be procured The absorption spectra of the extracts obtained and of a solution of the reference substance were recorded by a Beckman spectrophotometer (Model DK2)

Results and discussion

A preliminary recording of the absorption spectrum of the carotenoid material in iso-octane had indicated that β carotene was present In the final recordings of the spectra of the iso-octane extracts these spectra were therefore compared with a spectrum of synthetic β carotene dissolved in the same solvent As Fig 1 shows the spectrum obtained from the extracts of blood cells from adult cattle corresponds as regards the positions of the minimum and the maximum very closely with that of β carotene In relation to those in the spectrum of β carotene both maxima show an insignificant shift towards a shorter wavelength probably caused by the contamination with absorption, especially in the shorter wave part of the spectrum The extracts obtained with blood cells from sucking calves and pigs did not display any light absorption in visible light

A comparison between the absorption of the reference solution and that of the extract yields a β carotene concentration of 0 5 mg % in the blood cells which had been released from the chief part of the hemoglobin and treated with ultrasonic vibration

According to Bielig 1955 β carotene is present in the blood serum of cattle in a concentration of 0 023—1 35 mg % The procedure described above for isolating the

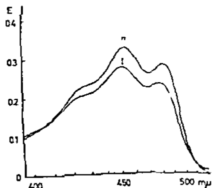


Fig 1 Absorption spectra (I) of fraction from cattle blood cells (II) of synthetic β carotene Solvent iso-octane

blood cell fragments involves a maximal dispersion of the whole mass of blood corpuscles of 15 times The total dilution of the remaining blood plasma must be very many times greater because the volume of the enclosed interstitial fluid is considerably reduced by the, at the centrifugations, considerable packing first of blood cells then of blood cells from which the main part of the hemoglobin has been removed and finally of disintegrated blood cells The greater part of the isolated β-carotene must thus derive from the blood cells

In cattle β-carotene clearly occurs not only in the blood plasma as stated in the literature (Hinsberg 1954, p 156 Bielig 1955 p 971, Rosio 1929), but also in the blood cells The erythrocytes of some birds fishes and reptiles are also stated to contain carotene (Zechmeister 1937) The β-carotene in the blood cells from adult cattle seems to derive from the fodder, since neither the blood cells from sucking calves nor those from pigs contained any measurable quantities of β-carotene

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Acta physiol scand 1971 83 4—12
From the Institute of Physiology, University of Bergen Norway

Electrolyte Distribution and Renal Function in the Hibernating Hedgehog

By

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Abstract

CLAUSEN, G and A STORESUND *Electrolyte distribution and renal function in the hibernating hedgehog* Acta physiol scand 1971 *83* 4—12

The concentrations of Na K Mg and Cl were determined in muscles and liver various zones of the kidney plasma red blood cells and bladder urine in active and hibernating hedgehogs Freezing point depression and urea concentration were determined in the kidney plasma and urine Unaltered tissue water concentration and 15 to 20 per cent decreased Cl and Na space suggest a shift of water from the interstitium to the cells during hibernation Hibernation tended to alter the partition of electrolytes between tissues and plasma similarly to *in vitro* cooling of mammalian tissues The bladder urine to plasma ratios of solute concentrations during hibernation were Na 0 18 Cl – 0 11 K – 6 4 Mg – 3 7 urea = 28 osmolarity = 3 4 Thus the extracellular ions Na and Cl are conserved relative to the intracellular ions during the hibernating season The osmolarity and the concentrations of urea, Na and Cl did not increase from cortex to papilla in the kidneys of hibernating hedgehogs Since the renal counter current multiplier system therefore is practically inoperative the hyperosmotic bladder urine (1 1 Osm/l) sampled during hibernation must be produced during the intermittent arousal periods and not during hibernation

During the hibernating season (November—April) the hedgehog neither eats nor drinks It spends about 80 per cent of the time in hibernation at about 5° C body temperature The hibernation is interrupted every one to two weeks by arousal periods during which the hedgehog rewarms to 34° C (Kristofferson and Soivio 1964)

The effects of hibernation on plasma and tissue electrolyte concentrations are not clear (for review see Kayser 1961 and Fisher and Manery 1967) The only invariate result seems to be that hibernation elevates plasma [Mg] Whether tissue [Mg] is altered is not known Most authors find unaltered or decreased tissue water concentration during hibernation (Willis 1967) Some investigators report increased plasma and tissue [K] while others find no alterations The [Na] data are in a similarly unsatisfactory state Plasma [HCO_3] is raised due to the greater CO solubility at lowered body temperature but otherwise the acid base balance of the blood is not significantly altered (Clausen 1966 Kent and Pierce 1967) Nothing further is

known of the anion concentrations in plasma or tissues during hibernation. Unfortunately, in most earlier reports, no tissue analyses accompanied those on plasma. Whether ionic gradients and water distribution between the inside and outside of tissue cells are maintained or not during hibernation, is consequently difficult to tell. We therefore determined the concentration of water, K and Mg, Na and Cl in plasma, various tissues and urine of active and hibernating hedgehogs.

The bladder urine sampled during hibernation is highly hyperosmotic relative to plasma (Clausen 1964). Pfeiffer and Moy (1868) found that the intra renal concentration gradients of urea and Na were abolished in hibernating ground squirrels. Further, the volume of bladder urine is not proportional to the duration of hibernation (Kristofferson 1965). These findings suggest that urine is not produced during hibernation but has been left in the bladder from the previous arousal period. However, several previous investigators (Hong 1957, Pengelly and Fisher 1961, Clausen 1964 and Kallen and Kanthor 1967) claim that urine is formed during hibernation. The intra renal solute concentrations and osmolarity indicate whether the kidneys produce hyperosmotic urine during hibernation. When hyperosmotic urine is formed the renal counter current multiplier system maintains a greater papillary than cortical and plasma osmolarity. A high papillary osmolarity is maintained only if glomerular filtration is taking place. A high papillary [Na] might be maintained without flow whereas a high urea concentration in the papilla requires flow in the collecting ducts and therefore also urine flow. If the urine found in the bladder during hibernation is produced during hibernation, the concentration of urea should be similar in the urine and in the tip of the papilla. Also, the osmolarity of the collecting duct fluid should be identical to that of the urine. We therefore investigatd the osmolarity and the concentrations of urea, C and Na at different levels from the papilla to the cortex of kidneys from active and hibernating hedgehogs.

Material and methods

The material consisted of adult hedgehogs *Erinaceus europaeus* L. The animals were kept in individual cages and fed a mixed diet of fish, bread and water. The hibernating group was kept in a refrigerated room at +3° C for at least a month before tissue and blood samples were taken. After 24 to 48 h deprivation of food and water, the active animals were anesthetized by i.p. injection of Nembutal Na (60 mg/kg b.w.) before sampling.

Blood, urine and bile

Blood was sampled from 15 active and 15 hibernating hedgehogs by catheterizing the ascending aorta, thus avoiding suction and preventing hemolysis that otherwise easily occurs when sampling from hibernating animals. Heparin (20 I.U. per ml blood) was used as anticoagulant. The blood was centrifuged at 3200 × *g* for 30 min at 5° C for hibernating and 38° C for active specimens.

The primary solution for red cells, plasma, bile and urine was 200 μl ad 10 ml deionized water. For Cl analyses 200 μl samples were deproteinized in 10 ml 1 % hydrated sodium wolframate in 1/12 N sulfuric acid.

Muscels and liver

Tissue samples were taken from 15 active and 15 hibernating hedgehogs. Pieces weighing about 300 mg of thigh muscle, diaphragma, left heart ventricle and liver were excised and blotted on filter paper before being weighed in platinum cubicles. The cubicles were gradually

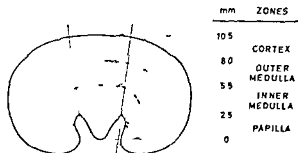


Fig 1 The zones of the hedgehog kidney and distances from the tip of the papilla referred to in the following figures

heated to 105° C and left at that temperature for 24 h before dry weight was determined The ashing was made after initial stepwise heating at 550° C for 15 h and at 650° C for 1 h White ash was obtained by oxidizing with 0 1 ml of concentrated nitric or hydrochloric acid drying and repeated heating

The primary solution for cation analysis was made by diluting the ash to 10 ml using 0 1 ml concentrated nitric acid and successive washings with deionized water For Cl analyses about 300 mg pieces were digested in 2 ml hot 1N potassium hydroxide for 30 min After neutralizing with sulfuric acid deproteinization was carried out at the same concentrations of wolframate and sulfuric acid as mentioned above

Kidneys

Renal osmolarity and solute concentrations were studied in 10 hibernating and 8 active hedgehogs The renal pedicle was clamped and the kidneys were removed shortly before the other tissues were excised. The midportion of each kidney was cut free and frozen in isopenthane cooled in liquid nitrogen The freezing point was determined by cryoscopy in 20 μ cryostat sections from one kidney as described by Wirz Hargitay and Kuhn (1951) The sections were made at five levels from cortex to papilla

The midportion of the second kidney was divided in its four zones (Fig 1) Pieces from each zone were treated according to Schmidt Nielsen and O Dell (1961) for subsequent Na Cl and urea analyses The water content was determined as described above

Analyses

[Na] [K] and [Mg] were determined by atomic absorption photometry (Perkin Elmer M 303) Four appropriate composite standards as well as a certified standard serum Seronorm Nyco A/S Oslo were run in each series of analyses Cl was determined by electrometric titration of 5 ml samples with 0 005 N silver nitrate (Radiometer pH meter PHM 27 GM and titrator TTT/a with a combined silver mercurous sulphate electrode type pK 499)

The freezing point of body fluids and renal tissue was determined by cryoscopy according to Ramsay and Brown (1955)

Urea was analysed according to Conway (1962)

Results and discussion

I Electrocyte concentrations of body fluids and tissues

The average electrolyte concentrations in active and hibernating hedgehogs are shown in Table I

Plasma

During hibernation plasma [K] and [Mg] were elevated by 40 and 50 per cent respectively Plasma [Na] had increased while [Cl] had decreased

The urea concentration of the plasma had increased from 20 3 mM in active to 23 8 mM in hibernating hedgehogs

TABLE I Electrolyte concentrations in tissues and body fluids of active and hibernating hedgehogs

	Na		K		Mg		Cl	
	Active	Hib	Active	Hib	Active	Hib	Active	Hib
Plasma	130.4	*143.3*	5.0	*7.2*	2.1	*3.3*	112.2	*102.3*
SD	±17.3	±10.6	±1.1	±2.7	±0.5	±0.6	±9.9	±12.0
Red cells	48.0	*70.1*	101.1	92.7	6.4	6.6	76.5	*88.7*
SD	±13.2	±10.1	±16.7	±11.0	±1.0	±0.7	±10.8	±13.9
Heart	51.5	*45.1*	111.2	*125.8*	26.2	*23.3*	65.5	*51.4*
SD	±7.5	±10.7	±17.9	±12.7	±7.1	±2.1	±19.3	±5.4
Diaph	43.6	40.0	139.2	141.5	31.8	*25.9*	56.5	*42.8*
SD	±9.5	±3.9	±20.4	±17.8	±7.4	±1.9	±15.8	±6.6
Thigh	37.5	35.8	119.2	*149.3*	25.6	*29.5*	60.0	*45.2*
SD	±7.0	±5.8	±15.1	±22.4	±4.6	±3.1	±15.9	±11.2
Liver	42.3	36.9	122.1	*138.3*	28.3	*26.0*	70.7	53.6
SD	±6.7	±2.7	±14.0	±5.3	±5.7	±1.3	±7.8	±13.6
Bile	152.9	169.3	8.5	7.0	6.5	*18.6*	32.1	*6.4*
SD	±24.9	±69.0	±2.3	±2.5	±4.4	±3.4	±22.8	±4.8
Urine	161.7	*26.6*	130.5	*46.3*	16.2	*12.1*	99.7	*12.1*
SD	±70.2	±16.6	±41.5	±27.6	±3.2	±4.0	±63.3	±12.1

Tissue and red blood cell concentrations are given as meq/l water plasma and urine concentrations as meq/l fluid. Red blood cell values are not corrected for trapped plasma. Concentration differences significant at the 5 per cent level or better are marked by italicized hibernation values.

Red blood cells

The red blood cells of active hedgehogs had 30 meq/l lower [K] and 30 meq/l higher [Na] than those of humans. The hedgehog thus occupies an intermediate position between typical high [K] and high [Na] erythrocyte mammals (*cf* Bernstein 1954).

Cellular [Na] and [Cl] had increased markedly during hibernation. The decrease of cellular [K] was, however, not significant ($P > 0.05$).

As shown in Table II the water concentration of red blood cells had increased by 5 per cent during hibernation. The cellular transmembrane potential, as calculated from the Cl partition between cells and plasma, was 9.3 mV during normothermia and 3.8 mV during hibernation. This agrees fairly well with the membrane poten-

TABLE II Water concentration (per cent of wet weight ±SD) of red blood cells and tissues in active and hibernating hedgehogs

Red cells		Heart		Diaphragma		Thigh muscle		Liver	
Active	Hib	Active	Hib	Active	Hib	Active	Hib	Active	Hib
66.7	*69.9*	76.7	76.9	75.6	74.7	75.1	*72.2*	71.0	70.8
±2.8	±1.9	±0.7	±2.4	±1.8	±2.7	±0.2	±1.4	±1.7	±2.8

Concentration differences significant at the 5 per cent level or better are marked by italicized hibernation values.

TABLE III The decrease of tissue Cl and Na space during hibernation in per cent The values in parantheses gives the decrease as per cent of tissue wet weight

	Heart	Diaphragm	Thigh muscle	Liver
Δ Cl space %	*14 (5 9)*	*17 (6 3)*	*20 (7 6)*	*14 (5 9)*
Δ Na space %	20 (6 4)	18 (4 7)	16 (3 8)	21 (5 0)

tials calculated from the pH of cell lysate and plasma derived from oxygenated blood at PCO_2 40 mm Hg (Clausen and Ersland 1969), showing that hibernation reduced the potential from 8 7 to 3 1 mV The electrolytes apparently have moved down their electrochemical potential gradient across the cell membrane and the cells have gained water This is similar to what occurs upon *in vitro* cooling of red cells and tissues from non hibernating mammals

Muscels and liver

The water concentration was not *sigfinicantly altered in liver, ventricle and* diaphragm, but had decreased by 4 per cent in the thigh muscle during hibernation compared to active controls (Table II) The [Cl] was lowered both in the tissues and in the plasma The intracellular [Cl] is only a small fraction of the extracellular [Cl] in mammalian muscle Since the fall of [Cl] in the tissues was even greater than in plasma, the Cl space of the tissues was markedly reduced during hibernation. The mean tissue Cl and Na space was calculated as

$$\text{Space \%} = \frac{\text{meq/kg tissue}\ \ 0{,}92\ \ \text{DF}\ \ 100}{\text{meq/l plasma}}$$

where DF (the Donnan Factor) is taken as 0 95 for Cl and 1 05 for Na The value 0 92 is a correction for the difference between plasma and extracellular water The reduction *of the Cl and Na spaces of the tissues during hibernation is shown in* Table III Since the water concentration of the tissues was unaltered (except in the thigh muscle) the reduced Cl and Na spaces may imply that the extracellular fraction of solvent water had decreased while the intracellular water had increased accordingly That the [K] had increased more in tissues than in plasma is consistent with such a shift of water The markedly decreased ratio of tissue to plasma [K] do indicate, irrespective *of the water shift that the [K] gradient across the cell mem* branes had decreased This, as well as the lowered body temperature per se would tend to lower the membrane resting potential during hibernation

The [Mg] of liver ventricle and diaphragm had decreased in spite of increased plasma [Mg] This is contrary to what might be expected since free intracellular Mg should behave like K *i e* tissue [Mg] should increase (*cf* Fisher and Maners 1967) However, the transmembrane gradient of [Mg] had decreased even more than that of [K] In the thigh muscle the increased [Mg] is probably due to the 4 per cent dehydration being mainly extracellular This is supported by the [K] having increased more in the thigh muscle than in the other tissues

In conclusion, hibernation tends to alter the water and electrolyte partition between intra- and extracellular compartments as does *in vitro* cooling of mammalian tissues.

Urine

The concentrations of all the electrolytes of the bladder urine were markedly lowered during hibernation. The osmolarity was 1.7 and 1.1 Osm/l and the concentration of urea was 900 and 630 mM/l in active and hibernating hedgehogs respectively.

The U/P (urine/plasma) ratios of hibernating hedgehogs were: Na = 0.19, Cl = 0.11, K = 6.4, and Mg = 3.7, showing higher renal retention of Na and Cl than of the mainly intracellular ions K and Mg during the hibernating season.

The [K]/[Mg] ratio is similar in the tissues and in the urine: 5 to 1 and 4 to 1 respectively. This agrees well with the obvious necessity for K and Mg excretion during the hibernating season when a substantial part of the body weight is being lost and intracellular ions are set free.

II. Renal water and solute concentrations

Water

The water concentration of the cortex and the outer medulla was not altered in hibernation. In the papilla the water concentration had increased by 6.3 per cent of tissue wet weight as seen in Table IV. The lowered papillary solute concentrations and the thus lowered tissue dry weight correspond to an increased water concentration of 6.6 per cent of tissue wet weight. This means that the hydration of papillary tissue, expressed as grams of water per 100 g non-solute tissue wet weight, was not altered by hibernation.

Osmolarity

As the frozen sections of renal tissue were slowly heated during the cryoscopy procedure the cellular content thawed first, followed by the tubular content. In the medulla and the papilla the smaller tubules (vasa rectae and/or loops of Henle) began thawing before the larger ones (collecting ducts). The large range of freezing point within the medullary and the papillary sections are probably due to artifacts as discussed by Bray (1960). The tubular freezing point depressions of active and

TABLE IV. Water concentration (per cent of wet weight ± SD) of renal tissue in active and hibernating hedgehogs

Cortex		Outer medulla		Inner medulla		Papilla	
Active	Hib	Active	Hib	Active	Hib	Active	Hib
78.2 ±0.5	77.9 ±0.9	81.4 ±0.8	81.7 ±0.8	83.2 ±1.9	*86.2* ±0.7	79.0 ±1.6	*85.3* ±0.5

Concentration differences significant at the 5 per cent level or better are marked by italicized hibernation values.

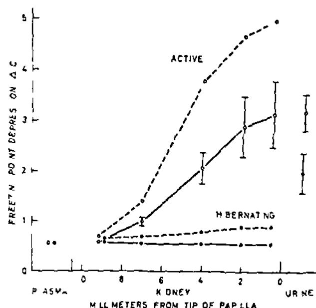


Fig 2 Freezing point depression in the kidneys of active (○) and hibernating (●) hedgehogs Mean values of first melting (broken lines) and last melting (solid lines) tubules are shown S D for plasma freezing point and for sections having freezing points close to that of plasma were too small to be presented in the figures (± 0 02° C)

hibernating hedgehogs are shown in Fig 2 The results from active specimens generally agree with those on dehydrated rats (Bray 1960) The osmolarity of tubular content increased gradually from the cortex to the papilla and the osmolarity of urine was within the range of that of the papillary collecting ducts

In the kidneys of hibernating hedgehogs the cortico papillary increase of osmolarity was practically absent However the urine osmolarity was more than 3 times higher than that of the collecting duct fluid There is no reason to assume that an intrarenal osmolarity corresponding to that of the bladder urine (1 1 Osml/l) should have vanished during the experimental procedure since it did not do so in the kidneys from active specimens We may therefore conclude that the hyperosmotic bladder urine of hibernating hedgehogs cannot have been produced during hibernation

Na and Cl concentrations

In active hedgehogs the [Na] increased from cortex to papilla as shown in Fig 3 During hibernation however no such intrarenal gradient of [Na] was found

The intrarenal [Cl] (determined in hibernating specimens only) was in accordance with the intrarenal [Na] (Fig 3)

Urea concentrations

The steeply increasing urea concentration from cortex to papilla of active hedgehogs (Fig 4) is similar to that of other mammals In hibernating hedgehogs the average urea concentration of the papilla was 33 8 mM *i e* only 7 mM higher than that of the cortex and 10 mM higher than that of the plasma The difference is not significant ($P > 0 05$)

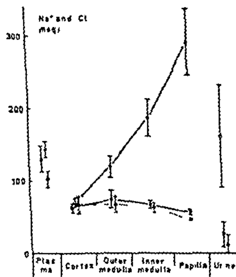


Fig 3

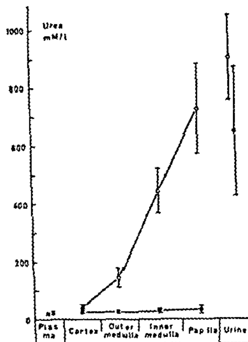


Fig 4

Fig 3 Sodium concentrations in the kidneys of active hedgehogs (O) and the corresponding concentrations of Na (●) and Cl (▲) of hibernating hedgehogs

Fig 4 Urea concentrations in the kidneys of active (O) and hibernating (O) hedgehogs

The urea concentration of the bladder urine sampled during hibernation was 650 mM, i e 19 times higher than that of the renal papilla

In conclusion, both the intrarenal osmolarity and the concentration of Na, Cl and urea show that during hibernation the counter current multiplier is not capable of producing the highly hyperosmotic urine found in the bladder of hibernating hedgehogs Most of the urine must therefore have been left in the bladder from the previous arousal period

The intrarenal Na and urea concentrations agree well with those found by Pfeiffer and Moy (1968) in hibernating ground squirrels Lesser *et al* (1970) found that urine flow did not begin in arousing ground squirrels until warmed up to at least 18° C During arousal the renal fraction of cardiac output is drastically (70 per cent) reduced (Soivio 1967) The sympathetic activation during the arousal process may cause preglomerular vasoconstriction and reduce the filtration pressure It is therefore not certain whether lack of urine flow during induced arousal also implies that urine flow is abolished during undisturbed hibernation The possibility that some urine slightly hyperosmotic or isomotic relative to plasma, is formed during hibernation cannot be excluded

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From the Brain Research Laboratory E-blocket and from the Department of Surgery University Hospital Lund Sweden

The DC Potential between CSF and Plasma in Respiratory Acidosis

By

Kenneth Messeter and Bo K. Siesjo

Received 8 January 1971

Abstract

Messeter K and B K Siesjo *The DC potential between CSF and plasma in respiratory acidosis* Acta physiol scand 1971 *83* 13—20

The electrical DC potential between cisternal cerebrospinal fluid and jugular venous blood was measured in rats during respiratory acidosis and alkalosis. In one of the main series studied respiratory acidosis and alkalosis was upheld for 5, 15, 45 and 180 min in animals anesthetized with nitrous oxide. In the other main series, sustained hypercapnia was induced by exposing unanesthetized rats to about 5 or about 11 % CO_2 for 3, 24 and 48 h respectively. In this series the animals were anesthetized during the last 30—45 min of the hypercapnia. In acute respiratory acidosis and alkalosis the CSF plasma potential difference varied linearly with the plasma pH with a slope of about -27 mV/pH unit. The CSF plasma potential differences measured in sustained hypercapnia did not deviate significantly from the mean regression line calculated from the acute experiments. The results thus showed that the CSF plasma potential difference was not only increased during acute respiratory acidosis but also remained increased as long as the plasma pH was acid.

There has been an increasing interest in the DC potential between cerebrospinal fluid (CSF) and plasma. This is mainly due to the fact that the potential may influence the distribution of cations and anions between CSF and plasma. Thus the fact that the CSF plasma potential varies with the plasma pH (Tschirgi and Taylor 1958, Mottshall and Loeschcke 1963, Held et al. 1964) suggests that the potential may be of importance in the regulation of the CSF pH in acute and sustained acid base changes. However an evaluation of the role played by the CSF plasma potential requires knowledge of the potential in both acute and chronic acid base changes. Recent experiments by Kjallquist (1970) (see also Goodrich 1965, Kjallquist and Siesjo 1967, 1968) have shown that the potential seems to be determined by the plasma pH also in sustained nonrespiratory acid base changes. However pure respiratory acid base changes were not studied, and the experiments were restricted to periods of six hours.

The present experiments were carried out to establish the relationship between the plasma pH and the CSF plasma potential difference (p.d.) in acute and

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The electrical DC potential between cisternal cerebrospinal fluid and jugular venous blood was measured in rats during respiratory acidosis and alkalosis In one of the main series studied respiratory acidosis and alkalosis was upheld for 5, 15, 45 and 180 min in animals anesthetized with nitrous oxide In the other main series sustained hypercapnia was induced by exposing unanesthetized rats to about 5 or about 11 % CO_2 for 3 24 and 48 h respectively In this series the animals were anesthetized during the last 30—45 min of the hypercapnia In acute respiratory acidosis and alkalosis the CSF plasma potential difference varied linearly with the plasma pH with a slope of about −27 mV/pH unit The CSF plasma potential differences measured in sustained hypercapnia did not deviate significantly from the mean regression line calculated from the acute experiments The results thus showed that the CSF plasma potential difference was not only increased during acute respiratory acidosis but also remained increased as long as the plasma pH was acid

There has been an increasing interest in the DC potential between cerebrospinal fluid (CSF) and plasma This is mainly due to the fact that the potential may influence the distribution of cations and anions between CSF and plasma Thus the fact that the CSF plasma potential varies with the plasma pH (Tschirgi and Taylor 1958, Mottshall and Loeschcke 1963 Held *et al* 1964) suggests that the potential may be of importance in the regulation of the CSF pH in acute and sustained acid base changes However an evaluation of the role played by the CSF-plasma potential requires knowledge of the potential in both acute and chronic acid-base changes Recent experiments by Kjällquist (1970) (see also Goodrich 1965, Kjällquist and Siesjö 1967 1968) have shown that the potential seems to be determined by the plasma pH also in sustained nonrespiratory acid-base changes However, pure respiratory acid base changes were not studied, and the experiments were restricted to periods of six hours

The present experiments were carried out to establish the relationship between the plasma pH and the CSF plasma potential difference (p d) in acute and

sustained respiratory acidosis in rats. To that end, the p.d. was measured both in anesthetized animals exposed to hyper- or hypocapnia for periods ranging from 5 min to 3 h, as well as in hypercapnic animals previously exposed to CO_2 containing gas mixtures in the unanesthetized state for 3, 24 and 48 h. The results showed a linear relationship between the plasma pH and the p.d. with a slope of about −27 mV/pH unit in both acute and chronic hypercapnia. A preliminary account of the results has been published (Messeter and Siesjo 1971 a).

Methods

The results were obtained on male rats of the Wistar strain weighing 300—450 g. The animals were anesthetized with divinyl ether, tracheotomized and artificially ventilated with a Starling type respirator (Braun, Melsungen), using a gas mixture containing 25—30 % oxygen and 65—70 % nitrous oxide. The animals were paralysed with tubocurarine chloride (Vitrum) in a dose of 0.2 mg/100 g i.p. The rectal temperature was measured continuously with a mercury thermometer and maintained close to 37° C by means of intermittent heating. Blood samples were collected and the blood pressure recorded from a cannula in the femoral artery. Arterial blood was analysed for pH, P_{CO_2} and P_{O_2} using micro electrodes (Radiometer, Copenhagen and Eschweiler & Co, Kiel) with appropriate corrections for body temperature. The pH values were referred to the phosphate buffers of the N.B.S. (pH 6.841 and 7.384, respectively).

The material consisted of two main groups. The first group was exposed to CO_2 concentrations of about 5 % and about 11 % respectively for 3, 24 or 48 h. These CO_2 concentrations were obtained by mixing CO_2, O_2 and N_2 by means of gas flow meters. The mixture was passed through a 100 l perspex box at a rate of 1.5 l/min and animal. During the exposure to the gas the animals were allowed access to water and to rat pellets. In order to determine the resulting arterial CO_2 tensions, arterial blood was repeatedly drawn from a tail artery in control experiments (see Pontén and Siesjo 1967 and Messeter and Siesjo 1971 b). At the end of the exposure period, the animals were taken from the box, anesthetized with divinyl ether and tracheotomized.

Immediately after the tracheotomy the previous CO_2-O_2 mixture was delivered to the respirator but now mixed with nitrous oxide instead of nitrogen. In order to achieve the same CO_2 tension obtained in the unanesthetized state, mechanical hyperventilation was performed. All the animals of this group were kept anesthetized for 30—45 min before the p.d. was measured. During this time, repeated arterial samples were taken for the control of pH, P_{CO_2} and P_{O_2}.

In the second main group anesthesia was induced as described above but the animals were exposed to an O_2-N_2O-N_2 mixture and the respirator was adjusted to give an arterial CO_2 tension of 35—40 mm Hg. When a steady state with regard to P_{CO_2} was obtained, groups of animals were then given about 11 % CO_2 or were mechanically hyperventilated and the p.d. was measured after 5, 15, 45 or 180 min, respectively. The hyperventilated groups were studied in order to facilitate calculation of $\Delta E/\Delta$ pH changes. *In all hypercapnic animals* mechanical hyperventilation was started at the time of the CO_2 administration.

The CSF plasma potential difference

The p.d. was measured between the cisterna magna and one external jugular vein using bridges of 3 M KCl in 2.5 % agar connected with saturated KCl calomel electrodes (K 100 Radiometer, Copenhagen) (see Kjallquist 1970). The p.d. measurements were performed with a differential amplifier connected to a potentiometric recorder.

At the beginning of the experiment, a polyethylene catheter (I.D. about 0.1 mm), containing the KCl agar gel, was inserted into the external jugular vein. The atlanto-occipital membrane was exposed just prior to the puncture. When the membrane had been exposed, a portion was scraped with a fine needle until only a thin layer remained. The glass pipette with KCl agar was lowered until it made contact with the membrane. Before penetrating the membrane with the pipette, the surrounding cavity was filled with liquid paraffine oil. A puncture was judged to be successful if the initial p.d. obtained remained stable within 2 mV during at least 5 min at a constant plasma pH, and if the potential difference did not change for small movements of the pipette. Likewise, the experiments were considered successful only if the asymmetry potential between the pipette and the catheter, as measured in a Krebs-Henseleit solution, remained within 1 mV during the experiments.

In the first part of the study somewhat larger glass pipettes were used In some of these experiments a leakage of CSF around the pipette could be observed This leakage was found to cause a decrease in the measured potential In order to minimize any error due to leakage the size of the pipettes was decreased to an outer diameter of about 60 μ (I D about 40 μ), and all experiments with a visible leakage were discarded

Results

The present results were all obtained in animals in which the arterial oxygen tension exceeded 90 mm Hg, the mean arterial blood pressure exceeded 120 mm Hg and the arterial hemoglobin concentration exceeded 14 g/100 ml The body temperature at the time of the p d measurements varied between 36 1 and 37 7° C

The experimental design aimed at keeping a similar arterial CO tension in the various subgroups This means that the plasma pH and the plasma bicarbonate concentrations varied with the time of exposure mainly due to compensatory renal mechanisms as shown in Fig 1 It should be noted that the plasma pH was still acid (pH 7 26) after 48 h of hypercapnia

Fig 2 shows the relationship between changes in the plasma pH and in the corresponding CSF plasma p d during acute exposures to about 11 % CO in the

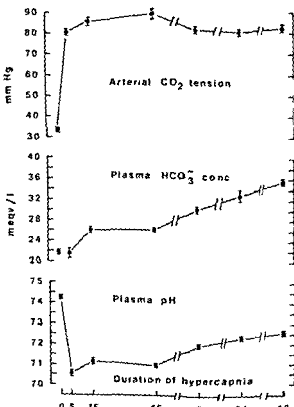


Fig 1 Measured arterial CO_2 tensions and pH values together with calculated plasma bicarbonate concentrations in groups of rats exposed to about 11 % CO_2 for various times (means ± S E) There were 3—7 animals in each group The figure illustrates the gradual increase in plasma pH due to an increase in the bicarbonate concentration However the plasma pH was not normalized after 48 h of respiratory acidosis

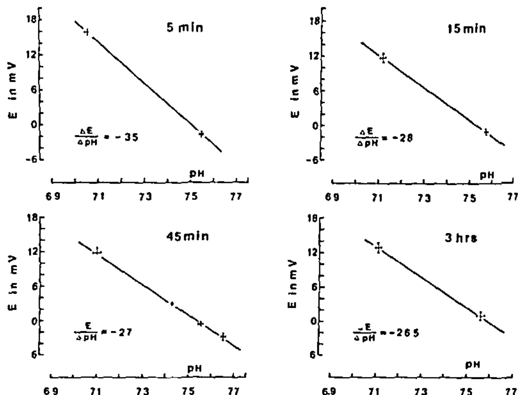


Fig 2 The relation between the measured plasma pH and the CSF plasma potential difference in anesthetized rats during respiratory acidosis and alkalosis Means ± S E The figure shows linear relationship between plasma pH and the potential difference An almost identical slope ($\frac{\Delta E}{\Delta pH}$) of about −27 mV/pH unit was found in the 15 and 45 min and in the 3 h groups

anesthetized state In order to determine the p d over a wide pH range normocapnia as well moderate and extreme hypocapnia was studied in the 45 min material The figure shows a linear relationship within the pH range of 7 1—7 6 The slope of the curve expressed as $\frac{\Delta E}{\Delta pH}$ was −27 mV/pH unit In the 15 min and 3 h groups respectively no significant changes in the $\frac{\Delta E}{\Delta pH}$ ratio could be detected ($p > 0.05$), whereas in the 5 min group the ratio was −35 mV/pH unit a significantly higher value in comparison to the other groups studied ($p < 0.05$) (see Discussion) The d c values obtained at the four exposure periods did not reveal any significant differences at a pH of 7 35 ($p = 0.12$)

The relationship between the plasma pH and the p d in sustained exposure of the rats to about 5 % or about 11 % CO_2 is shown in Fig 3 The line of regression (unbroken line), presented together with the 95 % confidence limits for estimated

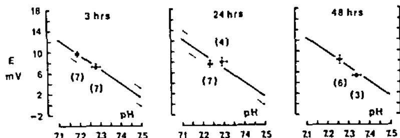


Fig 3 CSF plasma potential differences in anesthetized rats exposed to about 5 % CO_2 (filled circles) and about 11 % CO_2 (open circles) for 3 24 and 48 h respectively The figures within parentheses indicate number of experiments The estimated line of regression presented together with the 95 % confidence limits was calculated from all acute experiments including both hyper, hypo- and normocapnic values except those from the 5 min group (see Fig 2) The changes in the p d during sustained hypercapnia seemed to follow the plasma pH in the same way as in acute hypercapnia and the potential difference thus remained increased in the 24 and 48 h groups due to the persisting acidosis

values of E (broken lines), is a mean line obtained from all potential differences measured during acute respiratory acidosis and alkalosis, the 5 min group not being included The calculated slope of this line for the pH range of 7 1—7 5 was −27 mV/pH unit according to the equation E = −27 2 pH + 205 5 (r = 0 97), obtained from 38 experiments The hypothesis that the potential differences obtained during sustained hypercapnia followed the 'acute' $\frac{\Delta E}{\Delta pH}$ line was tested on the 5 % level for each individual measurement and could in no case be rejected This indicates a similar pH dependence as found in acute hypercapnia

Discussion

The present experiments had two main objectives The first objective was to study in rats the relationship between the plasma pH and the CSF plasma potential difference in acute respiratory acidosis and alkalosis the second to determine the p d in sustained respiratory acidosis The results confirmed previous results obtained in acute respiratory acid base changes in the dog (Held *et al* 1964, see also Sorensen Severinghaus 1970) in showing a linear relationship between the plasma pH and the p d with a slope close to −30 mV/pH unit In addition our experiments showed that the p d follows the plasma pH also in sustained respiratory acidosis In other words, the CSF plasma p d is increased not only in acute respiratory acidosis but remains increased as long as the plasma pH remains acid

Under the conditions of the present experiments *i e* during respiratory acidosis and alkalosis the changes in the measured CSF plasma p d seemed to be entirely determined by the plasma pH This was apparent from the fact that both the slopes and the intersects of the $\Delta E/\Delta pH$ lines at the various exposure periods were very similar, and that the p d values measured in sustained hypercapnia

(Fig 3) fell close to the mean regression line calculated for the acute experiments It has been reported that, in the cat, the CSF plasma potential differences correlate with the plasma bicarbonate concentration (Loeschcke 1971) Such a correlation did not exist in the present experiments Thus in sustained hypercapnia the p d decreased (see Fig 3) while there were increase in the plasma bicarbonate concentration (Fig 1)

As shown in Fig 2 there was a significantly steeper $\Delta E/\Delta pH$ slope in the 5 min group (about -35 mV) than in any of the other groups (about -27 mV) It is not clear if this difference has any biological significance since 5 min of hypercapnia represents such a nonsteady state with regard to pH and to the p d that it becomes difficult to assess the relation between the two Part of the difference between the 5 min group and the rest of the material may though be due to the fact that, in all other groups, there was at least one p d value considerably lower than the rest The aberrant values which were occasionally obtained in both hyper and hypocapnia could represent a partial shunting of the potential and would tend to give a $\Delta E/\Delta pH$ slope lower than the real one When evaluating the slope it should also be remembered that the real point of reference should be the mean capillary pH, which changes less during respiratory acidosis than does the arterial plasma pH For these reasons it seems appropriate to conclude that respiratory acid base changes are accompanied by $\Delta E/\Delta pH$ changes with a slope of about -30 mV/pH unit Further with the evidence at hand a calculation of the p d in all hypercapnic situations in the rat could be perfomed according to the following equation

$$E = +4\,0 - 27\ \Delta pH$$

where ΔpH is calculated as the deviation of the pH from 7 4

The p d is similar in dogs goats and rats (see Held *et al* 1964 Kjallquist and Siesjo 1967 Sørensen and Severinghaus 1970) The similarity does not only involve a similar absolute p d value at a plasma pH of 7 4 but also a similar slope in respiratory acid base changes This should make it possible to deduce the CSF plasma p d from the plasma pH in these experimental animals provided respiratory acid base changes are studied However it must be pointed out that this is not immediately permissible in nonrespiratory acid base changes which have been reported to give a larger $\Delta E/\Delta pH$ slope (Held *et al* 1964 see also Kjallquist 1970) or in cats and monkeys since acute experiments in these animals have shown that respiratory acidosis gives rise to p d in the opposite directions (Mottschall and Loeschcke 1963 Loeschcke 1970 Besson *et al* 1971)

The present results extend previous findings by Kjallquist (1970) in showing that the p d changes are upheld also in sustained respiratory acidosis However Kjallquist s results suggested a non linear relationship between the plasma pH and the p d The slope was calculated to about -30mV/pH unit in a plasma pH range within 7 2—7 4 and to about -50 mV/pH unit between 7 4 and 7 55 The difference in results does not necessarily represent an inconsistency, since Kjall

quist studied purely nonrespiratory or combined respiratory and nonrespiratory acid base changes, while the present results concern purely respiratory acidosis and alkalosis. Further, a subdivision of Kjällquist's data shows a linear relationship with a slope of approximately −35 mV/pH unit in combined respiratory and nonrespiratory acidosis, and a slope af about -42 mV/pH unit in sustained nonrespiratory acidosis and alkalosis, the latter value being in excellent agreement with that of Held *et al.* (1964). The slightly higher value in the first group compared to the results presented here could be due to the combination of nonrespiratory and respiratory acid base changes. However, it would seem that further experiments are needed to clarify the possible differences between sustained respiratory and nonrespiratory acid base changes (*cf.* Held *et al.* 1964).

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A note on the statistical analyses

1) The residual standard deviation σ of the E pH relationship during acute acid base changes (pH 7.1—7.5), was obtained together with the linear regression line given by the equation E = −27.2 pH + 205.5

$$\sigma = \sqrt{\frac{Q_0}{N-2}}$$

N = number of experiments

Q_0 = the residual sum of squares around the regression line

$$\sum_{i=1}^{38} (E_i - 205.5 + 27.2\ pH_i)^2$$

2) The regression line was used in order to obtain estimated E values in chronic hypercapnia with a standard deviation equal to

$$\sigma_1 = \sigma \sqrt{\frac{1}{N} + \frac{(\overline{pH} - pH)^2}{S_{pH}} + 1}$$

σ = the residual standard deviation of the E-pH relation in acute acid base changes

$\overline{pH}$ = the mean of the pH values on which the regression line was based, i.e. pH = 7.40

pH = the pH values obtained in sustained hypercapnia

S_{pH} = the sum of squares of the original pH values around their mean, i.e. $S_{pH} = 1.81$

3) A 95 % confidence interval for unknown E values in sustained hypercapnia was obtained from the estimated E value ± twice the standard deviation.

4) All statistical comparisons were made by use of Student's t test.

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Regulation of the CSF pH in Acute and Sustained Respiratory Acidosis

By

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Abstract

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In order to study the regulation of the cerebrospinal fluid pH during hypercapnia, plasma and CSF acid base parameters were measured in rats exposed to about 11 % CO_2 for periods varying from 5 min to 5 days. When combined with previously measured CSF/plasma d.c. potential differences, the plasma and CSF pH values and bicarbonate concentrations could be used to calculate electrochemical potential differences ($\Delta\mu$) for H⁺ and HCO_3^- between CSF and plasma. Hypercapnia lead to a 20 mEq/kg increase in the CSF bicarbonate concentration, most of which occurred during the first 24 h. There was a somewhat larger [HCO_3^-] increase in the CSF than in plasma water but in none of the compartments did the pH normalize. In acute hypercapnia (5—180 min) the calculated $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ values changed in a direction which would favor influx of HCO_3^- into the CSF. In the sustained groups (24—120 h) the $\Delta\mu$'s were close to the normocapnic control values. Since the accumulation of bicarbonate in the CSF occurred at a time when there was a suitable "passive" diffusion gradient, and since the CSF [HCO_3^-] in the chronic state was upheld without a significant change in the $\Delta\mu$'s, it is concluded that the regulation of the CSF pH in hypercapnia does not seem to require active transport of H^+ or HCO_3^-.

The pH of cerebrospinal fluid (CSF) has been shown to be remarkably stable in many chronic plasma acid-base disturbances. Although the deviations in pH from the normal values seem especially small in nonrespiratory acid base changes, and in respiratory alkalosis (Bradley and Semple 1962, Schwab 1962, Mitchell et al. 1965, Posner *et al.* 1965, Fencl *et al.* 1966), there is a significant regulation of the CSF pH also in respiratory acidosis. This regulation occurs through a gradual increase in the CSF bicarbonate concentration (Swansson and Rosengren 1962, Buhlman *et al.* 1963, Bleich *et al.* 1964, Ponten and Siesjo 1966 a) which eventually brings the CSF pH back close to normal values.

The mechanisms which allow an accumulation of HCO_3^- in the CSF during hypercapnia are not known. However, since the CSF essentially lacks nonbicarbonate buffers the extra HCO_3^- during hypercapnia must come either from the blood plasma or from the tissue. Recent experiments have shown that

capnia is accompanied by a gradual accumulation of bicarbonate in both the intra and extracellular space of the brain (Messeter and Siesjo 1970, 1971a, Siesjö and Messeter 1971) whence it appears unlikely that the accumulation in the CSF is due to a flux of bicarbonate from the cells

Although the bicarbonate accumulated in the CSF during hypercapnia probably emanates from the blood, the mechanisms involved have not been clearly defined However, it is known that the CSF HCO_3^- concentration does not passively reflect changes in the plasma [HCO_3^-] In the first instance, bicarbonate is not passively distributed in the electrical field between CSF and plasma (Severinghaus *et al* 1963, Held *et al* 1964) Secondly, in chronic respiratory and non respiratory acid-base changes in man the changes in the CSF bicarbonate concentration are only about 65 % and about 40 %, respectively, of the corresponding plasma changes (see Fencl *et al* 1966, Fencl 1971)

The nonequilibrium distribution of H^+ and HCO_3^- between CSF and plasma water suggests that one or both of the ions are being transported actively between the phases, and it seemed logical to assume that an active transport is responsible for the stability of the CSF pH (Severinghaus 1963, Mitchell *et al* 1965, Fencl *et al* 1966, Posner *et al* 1965) However, an active transport regulation of the CSF pH appears less likely since the electrochemical potential differences for H^+ and HCO_3^- between CSF and plasma remain essentially the same in sustained acid base changes (Siesjo and Kjallquist 1969, Kjallquist 1970)

In a preceding communication, we described changes in the CSF plasma potential differences in acute and chronic respiratory acidosis in the rat (Messeter and Siesjo 1971 b) In the present communication plasma and CSF bicarbonate concentrations were measured under similar experimental conditions The two sets of experiments enabled the calculation of electrochemical potential differences for H^+ and HCO_3^- and the differences will be used for a discussion of mechanisms regulating the CSF pH

Methods

General procedures The procedures were similar to those used in the preceding report (Messeter and Siesjo 1971 b) However in order to make it easier to obtain the intended CO_2 and O_2 concentrations the animals were anesthetized with 0 6 % halothane instead of 70 % N_2O All groups of rats were exposed to gas mixtures containing about 11 % CO_2 and 30 % O_2 In acute hypercapnia the gas mixture was administered to anesthetized animals for 5 15 or 45 min At the moment when the CO_2-containing gas mixture was administered mechanical hyperventilation was started (see below) In sustained hypercapnia unanesthetized animals were exposed to the gas mixture for 3 24 48 72 and 120 h respectively However in these groups anesthesia was given during the last 30—45 min During the anesthetic period the same gas mixture was delivered and the animals were mechanically hyperventilated so that the resulting arterial CO_2 tensions were close to those measured in the unanesthetized state These CO_2 tensions were controlled by repeated sampling of arterial blood from indwelling tail artery catheters (see Ponten and Siesjo 1967) in control animals exposed to the gas mixture in a Perspex box Controls were performed at 3 h (3 expts) 24 h (3 expts and at 72 h (1 [illegible] were within the range of 79 to 85 mm Hg [illegible] were males of the Wistar strain weighing [illegible] ether tracheotomized injected with tubo-curarine [illegible] The body temperature was maintained close

to 37° C One femoral artery was cannulated for blood sampling and for blood pressure recording

The blood samples were analysed for pH P_{CO_2} and P_{O_2} The values were corrected for differences in temperature between the animals and the electrodes (Severinghaus 1963) The pH values were referred to the phosphate buffers of the N B S (pH 6 841 and 7 384)

At the end of the experiments, and after that at least two sets of arterial samples had been taken a CSF sample was collected by puncturing the exposed atlantooccipital membrane with

10—15 min The plasma bicarbonate concentrations were calculated from the pH and the P_{CO_2} values The arteriovenous values thus obtained were used to calculate mean capillary pH values and bicarbonate concentrations (see below)

Calculation of acid base parameters and electrochemical potential differences The plasma bicarbonate concentrations (and the base excess values) were calculated from the alignment nomogram of Siggaard Andersen (1963), *i e* by assuming a CO_2 solubility factor of 0 03 mmole/l×mm and a pK 1 for carbonic acid of 6 10 The bicarbonate concentrations in plasma water were obtained by dividing the bicarbonate concentrations by 0 93 The mean capillary plasma pH values and the mean plasma bicarbonate concentrations were calculated as the arithmetic means of the arterial and venous values (see Results)

CO_2 tensions the latter being calculated as the arithmetic means of the arterial and the venous CO_2 tensions (see Results, and Ponten and Siesjo 1966 b) The pH of the CSF was calculated from the bicarbonate concentration and the CO_2 tension of the CSF, using a pK of carbonic acid of 6 125

Electrochemical gradients for hydrogen and bicarbonate ions were calculated according to the following equations

$$\Delta\mu_{H^+} = 61\ 5\ (pH_{pl} - pH_{CSF}) + E$$

$$\Delta\mu_{HCO_3} = 61\ 5\ \log \frac{(HCO_3)_{CSF}}{(HCO_3)_{pl}} \quad E$$

where $\Delta\mu$ is the electrochemical potential difference (in mV) and E the CSF plasma potential difference (also in mV) In the equations, pH_{pl} and $(HCO_3)_{pl}$ represent pH values and bicarbonate concentrations in mean capillary plasma water

The CSF plasma potential difference was derived from the relation between the arterial pH and the CSF plasma potential difference described in the previous communication (Messeter and Siesjo 1971 b) This relationship can be expressed by the equation E 4 0 − 27 ΔpH where ΔpH is the deviation of the plasma pH from 7 40 The standard deviation of this equation was also taken into account when calculating standard levels of estimated E and $\Delta\mu$ values

Results

A small series of measurements (6 animals) of the CSF plasma potential difference in animals anesthetized with 0 6 % halothane during acute and 'chronic (48 h) hypercapnia gave identical results to those reported previously for animals anesthetized with 70 % nitrous oxide Furthermore, a preliminary series of experiments on nitrous oxide anesthetized rats gave similar relations between the CSF and plasma bicarbonate concentrations in chronic' hypercapnia (21 and 48 h) For

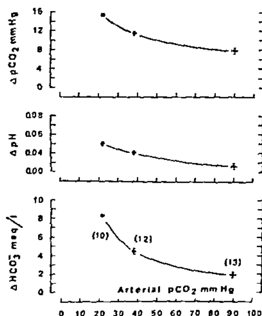


Fig 1

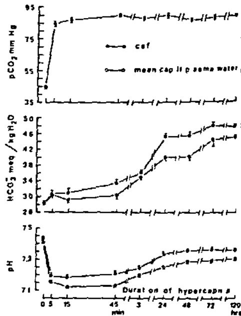


Fig 2

Fig 1 Differences in P_{CO_2} pH and $[HCO_3^-]$ between arterial blood and blood from the superior sagittal sinus in rats during normocapnia as well as during acute hyper and hypocapnia. The HCO_3^- concentration was calculated from measured values for pH and P_{CO_2} Means ± S E Number of measurements within parentheses

Fig 2 The CSF CO_2 tensions together with bicarbonate concentrations and pH values in CSF and in capillary plasma water of anesthetized rats exposed to about 11 % CO_2 for various iods Means ± S E The figure illustrates the gradual increase in both the CSF and the ean capillary bicarbonate concentrations as well as the corresponding changes in pH towards normocapnic values

these reasons the calculation of electrochemical potential differences seemed valid even if the values for the CSF plasma potential differences and for the CSF acid base parameters were obtained using different anesthetics

In order to allow a calculation of mean pH values and mean bicarbonate concentrations in capillary plasma water a series of control experiments were made with measurements of pH and P_{CO_2} in arterial blood and in blood from the superior sagittal sinus during normocapnia, and hypercapnia Fig 1 shows that in normocapnia the mean capillary plasma pH could be obtained by subtracting 0 020 from the arterial pH and the mean plasma $[HCO_3^-]$ by adding 2 mEq/l to the arterial bicarbonate concentration In hypercapnia the corresponding mean capillary values could be obtained by subtracting 0 005 pH units from the arterial pH and by adding 1 0 mEq/l to the arterial HCO_3^- concentration (see also Kjallquist 1970)

Table I gives the body temperature the arterial P_{O_2} and the directly measured acid base parameters in arterial blood in all the experimental groups In addition and in order to illustrate the gradual accummulation of bicarbonate during hyper capnia, the calculated plasma bicarbonate concentrations and the calculated base

[illegible]

Time of exposure	Body temperature	P_{O_2} mm Hg	P_{CO_2} mm Hg	pH	$[HCO_3]$ mEq/l	BE mEq/l
Control (9)	37 3 ±0 1	121 ±5	37 9 ±1 0	7 42 ±0 01	24 3 ±0 4	−0 5 ±0 3
5 min (4)	37 1 ±0 2	130 ±7	80 2 ±1 6	7 16 ±0 01	27 4 ±0 7	−3 6 ±0 7
15 min (5)	37 1 ±0 2	127 ±4	82 5 ±1 6	7 12 ±0 01	26 1 ±0 8	−4 8 ±0 6
45 min (5)	36 9 ±0 1	143 ±7	85 1 ±1 4	7 13 ±0 01	27 3 ±0 9	−3 8 ±0 7
3 hrs (5)	36 9 ±0 2	144 ±7	83 2 ±0 9	7 20 ±0 01	31 5 ±0 6	−1 2 ±0 6
24 hrs (4)	36 7 ±0	132 ±7	85 0 ±1 6	7 25 ±0 01	36 4 ±0 8	+6 0 ±0 7
48 hrs (5)	36 8 ±0 2	134 ±9	82 2 ±0 8	7 29 ±0 01	38 4 ±0 9	+8 6 ±0 7
72 hrs (4)	36 9 ±0 1	139 ±1	84 1 ±1 6	7 30 ±0 01	40 4 ±1 6	−10 0 ±1 4
120 hrs (4)	37 0 ±0 3	132 ±6	84 8 ±1 1	7 31 ±0 0	41 0 ±0 4	−10 6 ±0 2

excess values are also given The body temperature the mean arterial blood pressure (not given) and the arterial P_{O_2}'s were similar in all the groups There was a gradual increase in the plasma bicarbonate concentrations (and base excess values) during the hypercapnia and a gradual but incomplete normalization of the plasma HCO_3 concentration between the 72 and the 120 h groups ($0\,6 < p < 0\,7$ and $0\,7 < p < 0\,8$, respectively) whence it appears as if a steady state was reached after 72 h

Table II gives the mean values for the measured total CO content for the derived CO tension and for the calculated bicarbonate concentrations and pH values in cisternal CSF There were no *significant changes in pH or in the* $[HCO_3]$ between the 48 and 120 groups ($0\,6 < p < 0\,7$, and $0\,1 < p < 0\,2$ respectively) A comparison with the values of Table I suggests that there was a larger increase in the CSF than in the plasma bicarbonate concentration, and thereby a more pronounced normalization of the CSF pH

Fig 2 shows the derived CSF CO_2 tensions and compares the calculated values of CSF HCO_3 concentrations and the CSF pH with the corresponding mean capillary plasma values during the continuous state of hypercapnia The figure illustrates the gradual acid base changes in the two compartments and shows that there was a larger increase in the bicarbonate concentration of the CSF even if it was compared to the mean values for capillary plasma water

TABLE II Measured total CO_2 contents and calculated CO_2 tensions, bicarbonate concentrations and pH values in cisternal CSF for the same groups of animals as shown in Table I Means ± S E

Time of exposure	Total CO_2 mmole/kg	P_{CO_2} mm Hg	$[HCO_3^-]$ mEq/kg H_2O	pH
Control (9)	29 7 ±0 4	44 4 ±0 8	28 3 ±0 4	7 43 ±0 01
5 min (4)	33 4 ±0 9	84 7 ±1 5	30 7 ±0 9	7 19 ±0 02
15 min (5)	33 6 ±1 2	86 9 ±1 5	30 9 ±1 2	7 18 ±0 02
45 min (5)	36 3 ±0 9	89 6 ±1 0	33 5 ±0 9	7 20 ±0 01
3 hrs (5)	38 8 ±1 2	87 8 ±0 8	36 1 ±1 1	7 24 ±0 01
24 hrs (4)	48 2 ±0 6	89 4 ±1 5	45 4 ±0 7	7 33 ±0 01
48 hrs (5)	48 3 ±0 9	86 7 ±0 7	45 6 ±0 9	7 35 ±0 01
72 hrs (4)	50 8 ±0 8	88 0 ±1 5	48 0 ±0 8	7 36 ±0 01
120 hrs (4)	50 5 ±0 5	89 3 ±1 0	47 7 ±0 5	7 36 ±0 01

Fig 3 shows the changes in the ratio between the calculated HCO_3^- concentrations in CSF and in capillary plasma water during the hypercapnia. There was no significant decrease of the ratio at any of the exposure periods studied but a significant increase in the 24 h group In the other chronic groups (42, 72 and 120 h) the CSF plasma ratio was not significantly higher than in the control group ($p > 0.05$ but the direction of the changes in all groups suggested that the ratio remained increased in the chronic state

Fig 4 finally shows the changes in the estimated CSF-plasma potential difference (E), and the changes in the calculated electrochemical potential differences for H and HCO_3 $\Delta\mu_H$ and $\Delta\mu_{HCO_3}$ There were large increases in the $\Delta\mu$ values

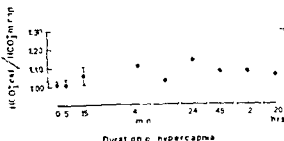


Fig 3 The ratio between the calculated HCO_3 concentrations in CSF and capillary plasma water during acute and sustained hypercapnia Means ± S E There was no significant decrease of the ratio at any of the exposure periods studied but a slight increase even in the sustained groups

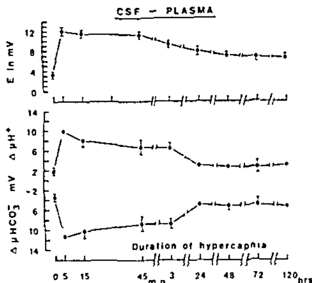


Fig 4 The derived CSF plasma potential differences (E) and estimated electrochemical potential differences for H^+ and HCO_3 ($\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3}$) during normocapnia, and during acute and sustained hypercapnia (see Methods) The figure illustrates the large increases in the $\Delta\mu$ values during acute respiratory acidosis In sustained respiratory acidosis $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3}$ returned towards the normocapnic levels although the estimated CSF plasma p d was still moderately elevated

in acute hypercapnia Since the CSF-plasma ratios for HCO_3 did not decrease during the hypercapnia (Fig 3) these were due to the (assumed) increase in the CSF-plasma potential difference In the chronic animals, the $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ values returned towards the normocapnic values The figures suggest that $\Delta\mu$ values were still slightly elevated in all the "chronic' groups (24—120 h) but a statistical comparison between each of the groups, and the control on the 5 % level, did not reveal any significant differences

Discussion

Before discussing the implication of the present results for current theories about CSF pH regulation it should be pointed out that the anesthetic procedure apparently affects the steady state relation between the CSF and plasma bicarbonate concentrations Thus although previous studies have shown that the CSF pH is more acid than the plasma pH in most animal species (see Fencl 1971) there was no significant difference in pH between CSF and mean capillary plasma in the present material This is apparently due to the anesthetic procedure which leads to a decrease in the plasma HCO_3 concentration of 2—3 mEq/l (Ponten and Siesjo 1967) However since anesthesia was induced in all hypercapnic groups it is probably safe to assume that the shift in the CSF plasma bicarbonate ratio, induced by the anesthesia was about equal in all groups and, therefore, that the influence of anesthesia does not affect conclusions regarding bicarbonate changes as a function of time

Degree of pH regulation in the CSF The present results have amply confirmed previous findings that sustained hypercapnia is accompanied by accumulation of bicarbonate in the CSF (see Introduction) However, our results indicate a more pronounced regulation than is apparent from previous work Thus, several clinical studies have shown that the increase in the CSF HCO_3 concentration during hypercapnia is only about 65 % of the corresponding increase in the plasma HCO_3 concentration (see Fencl 1971) In the present results there was an equally large, or even larger increase in the CSF than in the plasma bicarbonate concentration The results are more in accordance with a previous experimental study (Bleich *et al* 1964, see 5 day group) It is thus possible that previous clinical studies do not represent pure hypercapnia but that they reflect the CSF pH regulation in mixed conditions of hypercapnia and hypoxia (see discussions in symposium edited by Siesjo and Sorensen 1971)

Mechanisms of regulation Although the nature of the CSF plasma potential difference has not been defined it would seem logical to assume that it affects the distribution of ions between CSF and plasma We may thus use the electrical potential difference and the H or HCO_3 ratios between CSF and plasma for calculations of electrochemical potential differences and we seem entitled to interpret the presence of a net $\Delta\mu$ for H or HCO_3 as indicating active transport of the ions However we are then bound to use the same assumption in a changed acid base state such as hypercapnia *i e* we must assume that both the electrical potential difference and the chemical diffusion gradient influence the distribution of ions between CSF d plasma If the assumptions are valid we may evidently explain the increase in the CSF bicarbonate concentration during hypercapnia in purely passive terms Thus hypercapnia gives rise to a large electrochemical gradient favouring flux of HCO_3 from plasma to CSF Furthermore there is no further accumulation of HCO_3 in the CSF when the electrochemical potential difference decreases toward control values Finally the fact that in chronic hypercapnia the CSF bicarbonate increases more than the plasma bicarbonate is compatible with an increased CSF plasma potential due to a persisting plasma acidosis

As long as the nature and the significance of the CSF plasma potential difference are not known the exact mechanisms regulating the CSF pH will remain undefined However there seems to be no valid reason to assume that active transport of H or HCO_3 must be involved to explain the regulation of the CSF HCO_3 concentration in hypercapnia The rapid rate of accumulation of HCO_3 in the CSF during hypercapnia may require hydration of CO and formation of HCO_3 in the plexus chorideus and glial cells as proposed by Maren (1971) but as long as the transport mechanism cannot be shown to work against an electrochemical gradient it does not seem to be active in a thermodynamic sense

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The statistical analyses

1) Standard errors of the mean of the relatively small groups of estimated E values in sustained hypercapnia was calculated according to the following equation

$$S\,E_{E\ estim} = \frac{\sigma_1}{\sqrt{N_1}}$$

σ_1 = the residual standard deviation calculated from the E-pH relationship during acute acid base changes (see Messeter and Siesjo 1971 a)
N_1 — number of estimated E values

2) When calculating the standard errors of the estimated $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ the following equation was used

$$S\,E_{\Delta\ estim} = \sqrt{S\,E^2_{E\ estim} + S\,E^2_{\Delta\mu\ calc}}$$

$S\,E_{\Delta\ calc}$ = standard errors of the calculated values of $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ respectively

3) All statistical comparisons were made by use of Student s t test

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Postnatal Excitability Changes of Kitten Motoneurones

By

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Abstract

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The responses of lumbosacral motoneurones to iterative dorsal root (DR) stimulation were

studies on the postnatal changes in morphology and physiology of the cat spinal cord

The monosynaptic reflex which can be elicited in kittens even before birth, differs in many respects from that of the adult cat (Malcolm 1953, 1955, Skoglund 1960 a—e, Wilson 1962, Naka 1964 a, b Ekholm 1967) Immaturity of dorsal root collaterals and their terminals, differences in transmitter mechanisms, in permeability properties of motoneurone membrane and in the size of neurone somata and dendrites have been proposed as the underlying causes of differences in excitability conditions between the kitten and the adult cat (Skoglund 1960 d 1966 1967 Wilson 1962 Eccles Shealy and Willis 1963 Eccles and Willis 1963, 1965 Mellström and Skoglund 1969 Conradi and Skoglund 1969)

As earlier reported (Mellström and Skoglund 1969, Conradi and Skoglund 1969) there is a postnatal change in the size and synaptology of kitten motoneurones According to several authors (*cf* Henneman, Somjen and Carpenter 1965 a b Burke 1968 a, b) there is an inverse relation between the size and the excitability of neurones in the adult animal Against this background the postnatal changes in excitability of spinal extensor and flexor motoneurones were recently studied by

recording the monosynaptic reflex at different rates of iterative dorsal root stimulation (Mellström 1971 a). These and earlier studies indicate that there is a decrease in synaptic excitability with increasing age which might partly at least be due to the increase in motoneurone size. Differences in the stage of maturation in the monosynaptic pathway also exist between flexors and extensors at birth (Mellström 1971 a). Furthermore, the postnatal changes of recurrent effects were found to differ between flexors and extensors (Mellström 1971 b).

The purpose of the present investigation was to obtain intracellular information about some postnatally occurring excitability changes in ankle flexor and extensor motoneurones by studying the synaptic effects of iterative dorsal root stimulation. Postnatal changes in the magnitude of membrane resting potentials and in the characteristics of spike potentials were also investigated to serve as a basis for the interpretation of the intracellular responses obtained with iterative stimulation.

Material and Methods

The results of the present investigation were obtained by intracellular recording from 12[illegible] lumbosacral motoneurones collected from 26 kittens ranging in age from 1 to 70 days and from 5 adult cats.

Animals under ether anesthesia were spinalized between the first and second cervical vertebrae. The animals were then put on artificial respiration and immobilized by iv injection of Flaxedil®. A laminectomy was performed and the dorsal roots L4—S1 were cut on one side. The proximal parts of the cut L7 and S1 dorsal roots were mounted on a stimulating electrode. The ipsilateral tibial (extensor) and common peroneal (flexor) nerves were prepared for stimulation in the popliteal fossa. The spinal cord and the peripheral nerves were covered by mineral oil. The temperatures of the rectum and the oil pools were controlled by external heating and continuously recorded. The animals were rigidly fixed in a steel frame and usually [illegible] movements [illegible] were used for intracellular record[illegible] up to 20 MΩ. The microelectrode [illegible] motor micromanipulator (Tide and [illegible] by chlorided silver wires. The microelectrode was connected to a cathode follower from which the signal was fed into a d.c. amplifier, displayed on an oscilloscope and photographed on bromide film. The amplifier was also connected to a d.c. penwriter (Devices) for continuous recording of membrane potentials. A calibrator was inserted between the animal and ground in one of the arms of a Wheatstone bridge which allowed intracellular stimulation but in the work to be presented the bridge was only used for continually checking the resistance of the electrode when traversing the cord. The stimuli to the dorsal roots (DR) and peripheral nerves were square pulses of 0.3 msec duration, the frequencies used for iterative DR stimulation being 1/16 sec, 1/sec, 2/sec and 3.5/sec. The stimulus strength to the DR was, unless otherwise stated, kept well supramaximal for the monosynaptic response (approx. 10 × threshold).

Results

The impaled motoneurones were identified by their antidromic responses as belonging to the tibial (18 cells) or the common peroneal (37 cells) nerve but in kittens less than 20 days of age a large proportion of neurones, although located in the ventral horn, did not respond to antidromic stimuli. Many of these neurones were discarded, but others were not, since their monosynaptic spikes and general firing behaviour suggested that they were motoneurones. These neurones will be referred to as unidentified motoneurones (38 cells). The number of such unidentified cells

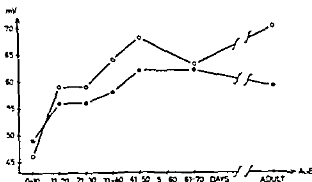


Fig 1 Mean values of membrane potential (●———●) and spike amplitude (○———○) plotted against age Each value is the mean from a varying number (9 to 23) of neurones Membrane potential is given in absolute values without signifying the inside negativity of the cells In each age group both spike amplitudes and membrane potentials have been obtained from the same motoneurones

decreased with age being 29 cells out of 46 before 20 days of age and only 9 out of 77 after 20 days No selection of impaled neurones was made on the basis of the value of their resting membrane potentials or spike potentials Only those neurones showing an unstable membrane potential or signs of deterioration were excluded

Membrane potential

The membrane potential measured as the potential difference between outside and inside was recorded as the sudden potential change occurring when impaling the cell and when leaving it In Fig 1 are plotted the mean values of the membrane potential measured at various ages in all cells, both identified and unidentified There is a general tendency for the membrane potential to increase in magnitude up to approximately one and a half months of age Especially in the youngest age group the mean value (taken from 23 measurements) was found to be rather low Since only neurones showing a stable resting potential and unaltered spikes for at least 30 sec have been used, it is not our feeling that membrane injury is the cause of the lower values in the young kittens On the contrary due to the postnatal increase of the cellbody volume (Mellstrom and Skoglund 1969) the difference might be even larger than indicated in Fig 1, since in the young kittens the microelectrode technique probably involves a preferential selection of the largest and most mature neurones with relatively high membrane potentials

Action potential

The duration of the action potential showed a slight tendency to decrease with increasing age of the kittens (Table I) No appreciable difference was found between the antidromic and ortodromic spikes in this respect The small neurones in the young kittens might be more vulnerable to electrode impalement and hence the longer spike duration could be due to cell damage Since many of the neurones however were kept for a long time without any change in spike appearance or resting membrane potential the observation might rather reveal different permeability properties of immature neurones The mean amplitude of the full action potentials also changed with the age of the kittens (Fig 1) being in general smaller in the younger kitte

TABLE I Duration of motoneurone anti- or orthodromic action potentials at different postnatal ages The figures for adult cats (antidromic potentials) are from Brock, Coombs and Eccles 1952

Age	Number of neurones	Mean in msec	Range in msec
0—10 days	23	1 8	1 3—2 9
11—30 days	31	1 5	1 2—2 0
31—70 days	34	1 3	1 0—1 6
Adult	13	1 0	0 85—1 25

Of considerable interest in this context is the finding that in the age group 0—10 days 18 cells out of 23 showed no overshoot, *i e* the majority of the cells did not reverse their membrane potential during the peak of the action potential This was also found, but to a much smaller degree (8 cells out of 25) between 11—30 days In adult cats, however, all neurones showed an overshoot as would be expected from the Hodgkin-Huxley theory Our results should be interpreted very cautiously although continuous checking of electrode resistance when traversing the cord did not indicate any major changes, which could help explain the lack of overshoot The membrane potential was measured as the difference between inside and outside, and the zero-line was frequently checked both during passage down the cord and also immedia ly after leaving a cell It is therefore our impression that the observation reflects t true situation although some individual values may be wrong in one direction or t other

In many motoneurones of kittens less than 40 days old it was found possible vary the amplitude of the orthodromic spike within a large range by varying t stimulus strength to the DR This is illustrated in Fig 2 from a 39-day old kitte The amplitude of the largest elicitable monosynaptic spike of this cell was equal that of the antidromic spike These reduced spikes were in several cases found to larger than the IS component of the antidromic spike of the same cell The signi cance of these spikes will be further considered in the discussion

Responses to iterative DR stimulation

The EPSPs evoked by DR stimulation in motoneurones of the youngest kittens we usually found to have a large amplitude (cf Eccles and Willis 1963) and they som

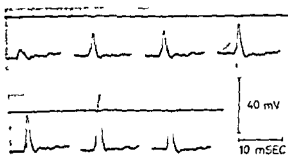


Fig 2 Intracellular recording of mono ... postsynaptic responses varying ... EPSP only (first sweep) to full spike last sweep) The reference line is on the same level in all sweeps

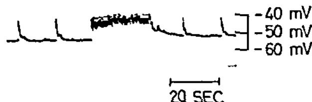


Fig 3 Intracellular recording from a tibial motoneurone in a 4-day old kitten Stimulus strength to the dorsal roots L7 and S1 supramaximal for the monosynaptic response (about 10 × threshold) The first two responses are elicited by single shocks at an interval of 16 sec Notice the long lasting depolarizations When stimulating the dorsal roots at 2/sec there is a sustained shift in membrane potential which disappears when stimulating at 1/16 sec again Spikes arising from the EPSPs not visible on this record

times lasted for as long as 1—2 sec (Fig 3) Although large variations in size of the EPSPs exist between motoneurones of different cats and even in the same animal there is a general tendency for them to decrease in both amplitud and duration with increasing age of the kittens When the EPSPs of extensor and flexor motoneurones were comparatively studied, they were not found to differ in any significant way Because of the long duration of many of these EPSPs recorded in the kittens only low frequencies of iterative dorsal root stimulation (*e g* 2/sec) were needed to make these potentials summate into large sustained depolarizations (Fig 3) When this occurs the amplitude of the EPSP has a tendency to decrease in proportion to the depolarization (*cf* Eccles and Willis 1963) As is seen in Fig 4 such sustained depolarizations are dependent upon both the frequency and the strength of the iterative stimuli, which indicates their synaptic dependency

In Table II and III and in Fig 5 are shown the changes in membrane potential caused by iterative DR stimuli at a frequency of 2/sec measured during the first 5 sec of stimulation on ankle extensor and flexor motoneurones in cats of different ages As a rule these effects did not change if the stimulation was maintained during

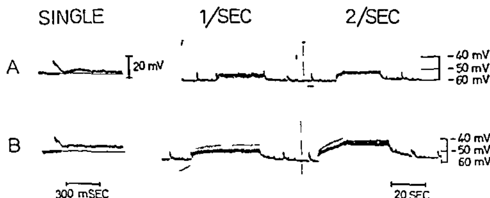


Fig 4 Intracellular recording from an unidentified lumbar motoneurone in an 1? day old kitten The dorsal roots L7 and S1 are stimulated just at threshold for the monosynaptic spike (A) and at 10 × threshold (B) A single shock causes a depolarization particularly long lasting in B Repetitive stimulation produces larger sustained effects in B than in A at both frequencies used The spikes arising from the EPSPs not visible in this record

TABLE II Amount of maximal polarization of ankle extensor motoneurones measured during the initial 5 sec of iterative dorsal root stimulation at 2/sec Depolarization and hyperpolarization are indicated by positive and negative signs, respectively

Age	Polarization in mV		Number of motoneurones			
	Mean	Range	Depolarized	Hyperpolarized	Non polarized	Total
0—10 days	+6 7	+1 4—+12 6	8	0	0	8
11—30 days	+4 8	+2 1—+ 8 4	6	0	0	6
31—70 days	+2 9	+1 4—+ 5 6	13	0	0	13
Adult	+0 6	−7 0—+ 4 9	10	6	5	21

TABLE III Amount of maximal polarization of ankle flexor motoneurones measured during the initial 5 sec of iterative dorsal root stimulation at 2/sec Depolarization and hyperpolarization are indicated by positive and negative signs, respectively

Age	Polarization in mV		Number of motoneurones			
	Mean	Range	Depolarized	Hyperpolarized	Non polarized	Total
0—10 days	—	—	—	—	—	—
11—30 days	+9 9	+7 0—+14 0	8	0	0	8
31—70 days	+4 3	±0— + 9 0	13	0	1	14
Adult	+2 7	−4 2—+ 6 3	13	1	1	15

some minutes In the kittens both flexor and extensor neurones responded only with depolarizations which were in general more powerful in ankle flexor than in ankle extensor motoneurones (Fig 5) With increasing age of the kittens, however, these depolarizing effects in mean gradually diminished, possibly due to combination of a decreased excitability for reasons mentioned in the introduction and the appearance of more inhibitory effects set up by DR stimulation Inhibition is especially evident in the case of the ankle extensor motoneurones in the adult cat where a strong hyperpolarization is often found in response to iterative DR stimulation (Table II)

Ankle flexor and extensor monosynaptic reflexes studied during repetitive dorsal root stimulation showed a somewhat different postnatal development (Mellström

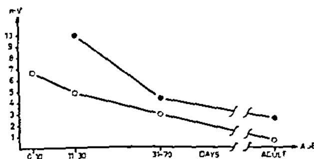


Fig 5 Mean membrane potential changes of ankle flexor (●——●) and ankle extensor (○——○) motoneurones caused by iterative dorsal root stimulation at 2/sec (ordinate) versus age (abscissa) Also compare Table II and III

TABLE IV *Number of ankle extensor and flexor motoneurones responding with a monosynaptic* spike to each dorsal root stimulus (firing index = 1 0) or not (firing index < 1 0) Stimulation frequency 2/sec Measurements are made both 0—5 sec and 25—30 sec after stimulation onset

Age	Time (sec) after stimulation onset	Extensor		Flexor	
		Fi = 1 0	Fi < 1 0	Fi = 1 0	Fi < 1 0
0—39 days	0— 5	14	2	11	0
	25—30	12	4	10	1
40 days — adult	0— 5	10	9	11	5
	25—30	8	11	13	3

1971 a) In the present investigation it was found that almost all ankle flexor and extensor motoneurones in kittens younger than 40 days responded with a monosynaptic spike on every dorsal root stimulus at a frequency of 2/sec, (Table IV) and mostly also at 3 5/sec *i e there was usually a monosynaptic firing index of 1 0,* which also remained during prolonged stimulation (*cf* Skoglund 1960 d 1966 and Wilson 1962) In older kittens and adult cats on the other hand, the motoneurones although always *responding with EPSP less often* gave monosynaptic spikes especially in the ankle extensor motoneurones during prolonged stimulation Furthermore, the ankle extensor motoneurones in kittens older than 46 days were more often found to respond only with an EPSP also at *the lowest stimulation frequency used (1/16 sec)* compared with ankle flexor motoneurones which always fired a monosynaptic spike in the spinal preparation used Although the material is too restricted to allow any conclusive statements about the small changes of firing probability which occur during prolonged stimulation (Table IV) they will be briefly dealt with in the discussion

In the youngest kittens the size of the monosynaptic spikes was in many cases related to the depolarization caused by iterative DR stimulation, meaning that the spike amplitude always decreased with increasing depolarization Some of these reduced spikes were quite small (less than 50 % of the maximal spike amplitude) and it remains to be solved whether such spikes are propagated down the axons or not

Discussion

The finding that many neurones here called unidentified but presumably motoneurones could not be invaded antidromically is in accordance with Naka s (1964 a) observation of the same phenomenon *Conradi and Skoglund (1969)* found the diameter of the motoneurone initial axon segment of the newborn kitten to be about half that of the adult animal whereas its length was the same This means that its *longitudinal* resistance would be about 4 times as high with a lowered safety factor for propagation as a consequence An increase with age of the number of motoneurones that are antidromically invaded would also be in good accordance with the

observation of a postnatal increase of antidromic effects on the monosynaptic reflex (Mellstrom 1971 b)

The action potential was found to have a slightly longer duration in the younger kittens As pointed out earlier this might be due to injured membranes in small cells but since stable membrane potentials could be recorded over a considerable time the observation might rather reveal different permeability properties of the immature motoneurone membrane Furthermore, the amplitude of the full action potential increased with increasing age Part of this increase appeared to be due to the gradual development of an 'overshoot or reversal of the action potential Factors like changing tip potentials etc might have given too high values of the membrane potentials which would explain the deficit in the spike However such changes in the microelectrode are likely to show themselves as changes of the electrode resistance but repeated checks thereof usually showed it to be constant We are therefore apt to consider this observation as a significant one which has also been obtained on kitten spinal ganglion cells (Kellerth and Skoglund to be published) The increase of the amplitude of the action potential with age should apart from changes in the conditions underlying the nonreversal of the spike be attributed to the postnatal increase of the membrane potential Such an increase is further evidenced by similar results in spinal ganglion cells of the kitten (Kellerth and Skoglund to be published) as well as in single cells in telencephalic structures of rat (Mares 1964) and in rat muscle cells (Fudel Osipova and Martynenko 1962 Boethius 1971)

The postnatal increase of the membrane potential might mirror different membrane permeability properties of immature neurones and reflect changes in the distribution of sodium and potassium across the cell membrane (cf Skoglund 1967) Changes of the latter type have been described in muscle cells during postnatal development (Vernadakis and Woodbury 1964 Hazlewood and Nichols 1969 Bergstrom Boethius and Hultman 1971)

In many cases variation of stimulus strength varied the size of the orthodromic spike potential The maximal orthodromic spike was however always of the same magnitude as the antidromic spike As shown by Kuno and Llinas (1970) in chromatolysed motoneurones summation of activation of dorsal root filaments gives gradation of the postsynaptic response which according to them might be due to summation of dendritic spikes The disappearance with increasing age of the spike variations with varying stimulus strength might be attributable to changing membrane properties but it could also be due to changing synaptological pattern The postnatal changes of motoneurone synaptology (Conradi and Skoglund 1969 presumably as a consequence of motoneurone growth (Mellstrom and Skoglund 1969) might involve a conversion of distal dendritic sections into more proximal ones Actually Conradi and Skoglund (1969 could not identify the typical monosynaptic bouton on soma or proximal dendrites in the newborn stage They might have overlooked these boutons which are few in number if present on more distal dendrites in the neuropil A postnatal change of the location of the monosynaptic boutons

from distal to proximal dendrites might underlie the disappearance with age of the spike potential variations with varying stimulus strength

As shown by Purpura, Shofer and Scarff (1965) a similar postnatal change in spike potential variation with stimulus strength also occurs in neonatal cortical neurones These authors assumed that the generation of "multiple-component' spikes occurred in the dendrites and that the postnatal changes thereof were due to profound alterations in the membrane properties This might, as pointed out above, be an alternative but not mutually exclusive explanation Voeller, Pappas and Purpura (1963) found axodendritic synapses on neocortical neurones to be numerous in the early stages whereas the axosomatic ones were few but increased especially during the second and third postnatal weeks when the rise time of EPSPs decreased In the present study the size of the EPSP of the motoneurones was found to decrease with increasing age (*cf* Eccles Shealy and Willis 1963) as in neocortical neurones (*cf* Purpura *et al* 1965), but since whole dorsal roots were stimulated nothing can be said about rise time and duration This postnatal decrease in size of the EPSP is a direct sign of a decreased excitability with age A causal relationship only between motoneurone size and excitability (Henneman *et al* 1965 a b, Burke 1968 a) during development, however, cannot be established without further experimentation Although a temporal correlation exists between the greatest and fastest changes in motoneurone size (Mellstrom and Skoglund 1969) and the changes in excitability, simultaneously occurring changes in synaptological pattern (Conradi and Skoglund 1969), electrolyte distribution, not to mention possible changes in membrane properties and transmitter actions might also contribute to the decrease in motoneurone excitability

Iterative dorsal root stimulation gave a shift in the membrane potential of the motoneurones, being greater in the younger stages and invariably a depolarization but in the adult animal the extensor motoneurones often showed a hyperpolarization or no shift at all With the frequencies used the motoneurones in the younger animal always gave a monosynaptic action potential, whereas in the older kittens and adult cats the motoneurones were not always able to follow the higher stimulation frequencies, especially 3 5/sec On the other hand, in the younger stages the orthodromic spikes suffered a reduction in size which in many cases was related to the depolarization of the cell The reduction of the spike seen with iterative stimulation thus appears to be a consequence of the depolarization and thus in its turn must mirror a difference in the transmitter action and/or membrane properties and/or motoneurone size and synaptological pattern of the immature neurone This can be concluded since in the adult stage the motoneurones no longer follow the stimulation frequencies and are usually not depolarized to the same degree as in the young kitten The variation of action potential size with varying stimulus strength on the other hand is as discussed above probably a consequence of dendritic spike summation

The results presented support and explain earlier observations (Mellstrom 1971 a) of a postnatal reduction of the effect of iterative DR stimulation Thus the EPSP the sustained depolarization and the firing index decreases with increasing age and

this explains the increase of the initial depression of the monosynaptic reflex with increasing age (*cf* Mellstrom 1971 a) The decreased depression of the monosynaptic potential in extensors with prolonged stimulation in the adult (*cf* Mellstrom 1971 a) points to an activation of a subliminal fringe as a consequence of presynaptic summation of activation The large EPSPs, the large depolarization and the high firing index would explain the small initial depression of the extensor monosynaptic reflex in the newborn stage The further depression seen with prolonged stimulation might be explained by the increased depolarization leading to a reduction in size of the spikes, some of which might not be propagated The different behaviour of the flexor monosynaptic potential being more depressed initially should then along the same lines be explained by a more advanced stage of development (*cf* Mellstrom 1971 a b c) and a less efficient synaptic drive in the flexor monosynaptic pathway The passing increase of this reflex in the early stages with prolonged stimulation might be explained by summation of depolarization and activation of a subliminal fringe The subsequent reduction would be a consequence of a further summation of depolarization giving nonprogated spikes The absence of an increase of the flexor reflex in the adult stage with prolonged stimulation would be explained by refractoriness due to the high activity of flexor motoneurones in the spinal preparation as evidenced by the unstable and small monosynaptic reflexes

For some of the explanations furnished above to be valid it still remains to be shown whether the reduced spikes are propagated or not The intracellular studies have provided some experimental explanation for the postnatal excitability changes taking place in the monosynaptic pathway So far their relative importance versus such in the recurrent pathway for the explanation of the postnatal changes of the recurrent effects has not been determined (see Mellstrom 1971 b) Obviously changes in the orthodromic activation play a role although further work is required to solve the question of possible postnatal changes in the recurrent pathway itself

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Changes in Continuously Recorded Intracranial Pressure of Conscious Rabbits at Different Time-Periods after Superior Cervical Sympathectomy

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Abstract

EDVINSSON, L., CH. OWMAN and K. A. WEST. *Changes in continuously recorded intracranial pressure of conscious rabbits at different time-periods after superior cervical sympathectomy.* Acta physiol. scand. 1971. 83. 42—50.

The intracranial pressure, measured as the ventricular fluid pressure (VFP), was recorded continuously during about 2 days via a cannula inserted into the left lateral ventricle of the brain of conscious rabbits. The effect of bilateral removal of the superior cervical ganglia on the VFP was studied at various time-periods after operation, and the results were compared with those from unoperated control animals. The pressure changes attributed to the sympathectomy are referred to as the net VFP. The operation ultimately caused a disappearance of noradrenaline from intracranial sympathetic nerves. The net VFP was not affected during an 8-hr period of the recording starting 5—8 hrs after sympathectomy. During the following 35 hrs it was reduced by approximately 25 mm physiological saline followed by a return to initial or somewhat higher levels. Four days after sympathectomy the net VFP was significantly increased throughout the recording period. Two weeks after the operation the pressure had returned to the same or even lower level compared with the non-sympathectomized control animals. The variations in the net VFP at different time-periods after sympathectomy are considered to reflect mainly changes in the intracranial vascular bed due to the leakage and disappearance of the noradrenaline transmitter from the degenerating nerve terminals followed by denervation supersensitivity. The results are discussed in terms of a sympathetic influence on the intracranial pressure mediated through the volume of the intracranial vascular bed, and/or the cerebrospinal fluid production in the choroid plexuses.

The normal level of intracranial pressure is primarily maintained and regulated through a combination of changes in the cerebral blood flow and in the circulation of the cerebrospinal fluid (CSF). It has been shown that two components in this regulatory mechanism receive a sympathetic nerve supply. Thus, the main pial vessels, particularly the arterial system, possess a rich to very rich adrenergic innervation (Nielsen and Owman 1967, Spoendlin and Lichtensteiger 1967, Donath 1968, Falck et al. 1968, Kajikawa 1968, 1969, Ohgushi 1968), and a substantial amount of adren-

ergic nerves has also been demonstrated in the choroid plexus (Edvinsson *et al* 1971 c) These findings have offered a structural basis for further functional studies on the possible influence of the sympathetic nervous system on the intracranial pressure

It has recently been found that bilateral removal of the superior cervical ganglia, which produces a sympathetic denervation of the pial vessels and the choroid plexuses (Nielsen and Owman 1967, Edvinsson *et al* 1971 c), to a high degree prevents the development of intracranial hypertension induced by cisternal injection of kaolin (Owman and West 1970) and changes in cerebral blood volume at various time periods after the sympathectomy (Edvinsson *et al* 1971 b) In order to allow a more detailed analysis of factors influencing the intracranial or ventricular fluid pressure (VFP), a technique was devised for continuous recording of the ventricular fluid pressure in conscious rabbits over a period of up to 80 hours (Edvinsson *et al* 1971 a) In the present investigation this technique has been utilized to record variations in the VFP as a consequence of previous superior cervical sympathectomy and to study any difference in the effect when the denervation was carried out at different time periods before the pressure recordings

Material and Methods

The experiments were performed on 24 rabbits of either sex weighing between 2.3 and 3 0 kg and fed freely with standard pellets (SAN bolagen Sweden) turnips and tap water The animals were sympathectomized by bilateral removal of the superior cervical ganglion A first group of 10 animals (Group 1) was sympathectomized under light dinitrous oxide halothane (Fluothane I C I) inhalation anesthesia and two other groups (Group 2 8 animals and Group 3 6 animals) under Nembutal anesthesia (1 8 ml of a 6 % solution diluted in physiological saline and administered intravenously) Five to eight hours (Group 1) 4 days (Group 2) or 14 days (Group 3) after sympathectomy a pressure cannula (Owman and West 1970) was inserted under local anesthesia (3 ml of 2 % lidocain Xylocain Astra Sweden) into the left lateral ventricle of the brain The subsequent recordings of the VFP were performed on a Grass Model 7 Polygraph via a Statham Model P23AC transducer as earlier described in detail (Owman and West 1970 Edvinsson *et al* 1971 a)

It has previously been reported (Edvinsson *et al* 1971 a) that implantation of the pressure cannula into the brain of normal rabbits itself produces an increase in the VFP probably as a result of a local traumatic brain edema probably combined with a disturbance in CSF absorption On the basis of these alterations in the VFP which have previously been discussed in detail a methodological base curve was constructed (see Edvinsson *et al* 1971 a) Since these recordings were carried out in the course of the present study the animals will be regarded as the *control group* This control group allows correction of the presently obtained pressure curves for that increase in the VFP induced by the direct recording technique used The correction is made by subtracting the sequence of mean pressure levels in the control group from the corresponding time related mean pressure levels in the 3 different groups of sympathectomized animals The resulting pressure which thus reflects the specific effect of the sympathectomy is called *the net VFP*

Differences between separate mean values in the pressure curves were analyzed with the Student s *t* test A possibly significant sloping tendency within the curves was further evaluated statistically by estimation of the regression coefficient and construction of the corresponding regression line (Bonnier and Tedin 1957)

... air 2 days ... arteries at the ... desiccator in the ... dehyde gas at 80° C during 1 hr for the fluorescence histochemical demonstration of the noradrenaline transmitter in the sympathetic nerves (Falck and Owman 1965)

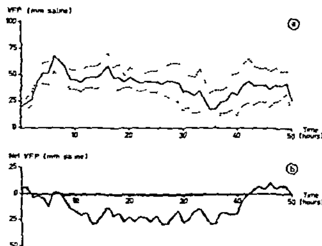


Fig 1 (a) Mean VFP from recording on animals 5—8 hrs after sympathectomy Mean ———, ± standard error of the mean (b) Mean net VFP, obtained by subtracting the value at each time interval in the methodological base curve (Edvinsson *et al* 1971 a) from the corresponding time related values in the curve of Fig 1 a in animals 5—8 hrs after sympathectomy

Results

Bilateral removal of the superior cervical sympathetic ganglia ultimately resulted in *the disappearance of noradrenaline from all sympathetic nerves supplying the main* pial arteries and the choroid plexuses showing that the sympathectomy was complete.

Group 1 VFP 5—8 hrs after sympathectomy

The mean VFP as obtained from continuous recordings during about 50 hrs in conscious animals subjected to bilateral superior cervical sympathectomy 5—8 hrs before insertion of the ventricular cannula is shown in Fig 1 a The recording system remained patent in 7 of the animals throughout the entire 50-hr period For technical reasons no pressure could be obtained in 3 of the animals at various periods between 22 and 50 hrs

The initial VFP measured as the mean pressure during the initial half hour after insertion of the cannula was 20 mm physiological saline During the first 10 hrs the mean pressure showed a transient increase to a maximum of approximately 70 mm saline Subsequently it remained fairly constant on a level of about 40 mm saline except for a transient dip at 35 hrs

As seen from Fig 1 b the mean net VFP calculated as described in Material and Methods remained essentially unchanged (*i e* the pressure was at the same level as in non-sympathectomized animals) during the first 8 hrs of the recording During the following period of almost 35 hrs the net VFP was reduced by approximately 25 mm physiological saline The mean pressure then returned to a level that was the same or slightly higher compared with the control animals

Group 2 VFP 4 days after sympathectomy

In this group the recording system remained patent in all 8 animals during the first 20 hrs. During the remaining period (up to 42 hrs) recordings could be obtained

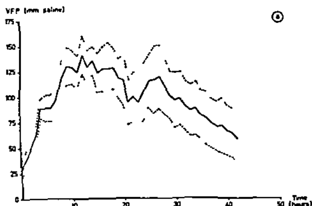




Fig 2 (a) Mean VFP from recordings on animals 4 days after sympathectomy Mean ———, ± standard error of the mean - (b) Mean net VFP (methodological base curve subtracted from curve in Fig 2 a) in animals 4 days after sympathectomy

only from 5 of the animals Fig 2 a shows that the initial mean pressure was at 28 mm saline The mean VFP then increased to 130 mm during the first 10 hrs, followed by a slow, continuous decrease to about 60 mm saline at the end of the recording period

Fig 2 b illustrates the alterations in the net VFP (calculated by subtracting the mean pressure levels in the non sympathectomized control group from those time-related values obtained in the group of sympathectomized animals) It is evident from the figure that the mean net pressure was significantly increased throughout the recording period During the first 10 hrs the net VFP increased rapidly from 15 mm to 70 mm physiological saline In the following 30 hrs the net pressure showed a steady decrease until the initial level was reached As evaluated from the slope of the calculated regression line, this tendency in the continuous reduction of the net VFP is at the highest level of significance

Group 3 VFP 14 days after sympathectomy

Fourteen days after bilateral ganglionectomy the initially recorded pressure was 16 mm saline The subsequent mean VFP values were obtained from recordings on 6 animals In one of these animals the recording system remained patent only for 6 hrs Also in this group of animals (Fig 3 a) the total VFP rapidly increased during the first few hours after implantation of the pressure cannula, and a maximum level of approximately 100 mm physiological saline was reached after 4 hours The mean VFP then decreased to about 60 mm saline This level was essentially constant

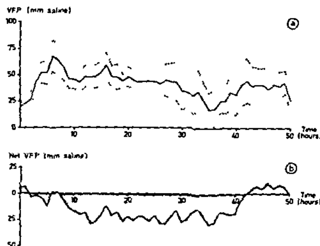


Fig 1 (a) Mean VFP from recording on animals 5—8 hrs after sympathectomy Mean ———, ± standard error of the mean (b) Mean net VFP, obtained by subtracting the value at each time interval in the methodological base curve (Edvinsson *et al* 1971 a) from the corresponding time related values in the curve of Fig 1 a in animals 5—8 hrs after sympathectomy

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Group 2 VFP 4 days after sympathectomy

In this group the recording system remained patent in all 8 animals during the first 20 hrs During the remaining period (up to 42 hrs), recordings could be obtained

the total VFP at the various time intervals in the sympathectomized animals The resulting curves thus express the net VFP, *i e* the changes occurring in the VFP as a consequence of the superior cervical ganglionectomy This net VFP showed a sequence of variations during different time periods after the ganglionectomy Thus, shortly after the operation the net VFP was initially unchanged and then during 30 hrs reduced by about 25 mm physiological saline Approximately 4 days post operatively the net VFP was increased by 70 mm saline but a progressive reduction could be seen and 2 weeks after sympathectomy the net VFP was at the same or slightly lower level compared with the VFP in the unoperated controls

It is well known that an increased volume of the intracranial vascular system increases VFP although this is soon compensated by lowered CSF volume The pial contribution to this vascular system has been found to receive an ample sympathetic innervation (Nielsen and Owman 1967 Spoendlin and Lichtensteiger 1967, Donath 1968 Falck *et al* 1968 Kajikawa 1968 1969 Ohgushi 1968) The functional significance of the neurogenic vasomotor control of the pial vessels is poorly understood At least the ability of the cervical sympathetic system to produce a vasoconstriction of the vessels upon stimulation is well demonstrated (Sokoloff 1959 Kety 1960 Sears and Bárány 1960 Klosovskii 1963 Rosenblum 1965 Lazorthes *et al* 1966) This agrees with the recent observation that noradrenaline (Nielsen and Owman 1971)—as well as tyramine (Nielsen *et al* 1971) which acts by releasing endogenous noradrenaline from the perivascular sympathetic nerves—causes a contraction in isolated pial arteries It is thus possible that the observed alterations in the net VFP curves as a consequence of the ganglionectomy at least partly reflects changes in the intracranial blood volume (*cf* Edvinsson *et al* 1971 b) which are not compensated by the CSF system if absorption of CSF is impaired (Edvinsson *et al* 1971 a)

During approximately the first 8 hours following sympathectomy the noradrenaline content in sympathetically innervated organs remains virtually unchanged (Weiner *et al* 1962 Benmiloud and Euler 1963) which would agree with the present finding that the postoperative net VFP was initially about zero Subsequently there is a leakage of stored transmitter from the sympathetic nerve terminals which is accompanied by an activation of the effector structures (Sears and Barany 1960 Coats and Emmelin 1962 Langer 1966 Emmelin and Ohlin 1969 Lundberg 1969) for about one day During a corresponding time period the net VFP was found to be significantly lowered at least partly as a consequence of a vascular constriction (and reduced intracranial blood volume) caused by the leaking noradrenaline (*cf* Edvinsson *et al* 1971 b) Moreover it has recently been shown (West 1971) that acute sympathectomy reduces the development of traumatic edema (*i e* that produced by implantation of the cannula in the present experiments)

Four days after sympathetic ganglionectomy the transmitter has completely disappeared from the degenerating nerve endings (Malmfors and Sachs 1965 Smith *et al* 1966 Van Orden *et al* 1967) and as a result of the loss of the vasoconstrictor nerves, the brain vessels are expected to dilate (*cf* Edvinsson *et al* 1971 b) thus increasing

the volume of the vascular bed. Provided this is not compensated, it would be in agreement with the higher level of the VFP noted at this period compared to the VFP of unoperated control animals. During the first hours after insertion of the pressure cannula in this group of sympathectomized animals, the net VFP was at a relatively low level (although the mean pressure was still significantly higher than in the control animals). This could be due to a stress-induced (insertion of the pressure cannula) increase in circulating catecholamines which constrict the sympathetically denervated vessels having reached a rather pronounced degree of supersensitivity to catecholamines already at this postoperative stage (Langer *et al.* 1967). During the remaining part of this recording period the net VFP showed a progressive and significant decrease. This conceivably reflects a continuously further enhanced denervation supersensitivity (Langer *et al.* 1967) which will tend to increase the vascular tone—and thus reduce the intracranial blood volume and the VFP—also under the influence of the normal level of circulating catecholamines.

It has been demonstrated by using the nictitating membrane from pithed cats that the sensitivity to catecholamines is increased by almost thousand times within 2 weeks following sympathetic denervation of the membrane (Langer *et al.* 1967). This could well explain the present observation that the VFP during the main part of the recording period 2 weeks after ganglionectomy was at the same level or even lower than that in unoperated control animals: the sympathectomized intracranial vessels have probably reached the same—or even a higher—degree of tone compared with the controls (*cf.* Edvinsson *et al.* 1971 b).

It has previously been found in unoperated animals (Edvinsson *et al.* 1971 a) that implantation into the brain of the pressure cannula produces a local edema, which is conceivably the main reason for the increase in VFP that builds up in such animals within the first 10 hrs after implantation. It is possible that the expected increase in circulating catecholamines in connection with this implantation (also discussed above) will cause a pronounced and transient intracranial vasoconstriction in the 2 week group of sympathectomized animals because of the very marked supersensitivity at this postoperative stage (Langer *et al.* 1967). This vasoconstriction may be the underlying mechanism for the aggravation of the traumatic edema shown to occur after sympathectomy (West 1971); it may therefore account for the increase in the net VFP observed during the first 10 hrs after implantation of the cannula in this group of animals. When the level of circulating catecholamines then normalizes and the pronounced vasoconstriction subsides, the edema diminishes to the level seen in the non-sympathectomized control animals.

It has been found that the choroid plexuses, which are responsible for part of the CSF production (Davson 1967), receive an adrenergic nerve supply from the superior cervical sympathetic ganglia (Edvinsson *et al.* 1971 c). Some of these nerves are vascular and may thus have a vasoconstrictor function. Hence, it can be expected that the ganglionectomy will induce a similar sequence of changes in the plexus vessels as in the pial vessels, *i.e.* vasoconstriction followed by dilation and recovery of tone. It can be assumed that arterial vasoconstriction in the choroid plexuses will

reduce CSF production Following sympathectomy the variations in the plexus blood circulation that may affect the CSF production will therefore add to those vascular changes that alter VFP through an effect on the intracranial blood volume

It was earlier observed (Owman and West 1970) that bilateral superior cervical ganglionectomy 4—8 days before kaolin induced blockade of the CSF efflux will markedly reduce CSF production as a possible consequence of the loss of secretory nerves It can be assumed that any such effect of sympathectomy has had little influence on the VFP in the present experiments because they have probably been masked by the vascular changes discussed above and, moreover, they would be expected to counteract these changes

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Modification of Kaolin-Induced Intracranial Hypertension at Various Time-Periods after Superior Cervical Sympathectomy in Rabbits

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Abstract

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The intracranial pressure measured as the ventricular fluid pressure (VFP) was recorded continuously via a cannula in the left lateral ventricle of the brain of conscious rabbits 2 days after intracisternal administration of kaolin, known to induce intracranial hypertension. The

sympathectomy the pressure tended to be lower than in the non-sympathectomized control animals. It is suggested that these findings are mainly the result of changes in the intracranial blood volume (although direct effects of the sympathectomy on cerebrospinal fluid circulation can not be excluded). Thus, shortly after sympathectomy—period (a)—when the transmitter

the brain vessels have developed a pronounced denervation supersensitivity to circulating catecholamines, the vascular tone is enhanced and the reduced blood volume will tend to lower the VFP.

It has recently been shown that bilateral excision of the superior cervical sympathetic ganglia produces marked alterations in the ventricular fluid pressure (VFP) as recorded continuously during more than 2 days in conscious rabbits (Edvinsson *et al.* 1971 c) via a cannula implanted into the left lateral ventricle of the brain (Owman and West 1970). These findings indicate that the cranial sympathetic nervous system somehow influences the two major mechanisms—the cerebral blood volume,

cerebrospinal fluid circulation—effecting the intracranial pressure. Moreover, the results demonstrated that the effects of *sympathectomy were considerably different* at various time periods after the operation (Edvinsson *et al.* 1971 c). The differences were most probably related, first, to the release (Lundberg 1970, Edvinsson *et al.* 1971 b) and disappearance (Malmfors and Sachs 1965, Smith *et al.* 1966, Nielsen and Owman 1967, Van Orden *et al.* 1967, Edvinsson *et al.* 1971 b) of the noradrenaline *transmitter from the degenerating perivascular sympathetic nerve endings* and, subsequently, to the progressive development of denervation supersensitivity of the catecholamine receptors (Langer *et al.* 1967, Edvinsson *et al.* 1971 b). As a consequence of this, a normal or almost normal VFP was restored already within 2 weeks after sympathectomy (Edvinsson *et al.* 1971 c).

The *injection of kaolin into the cisterna magna* is a widely used experimental technique for producing hydrocephalus and intracranial hypertension. These conditions are considered to be the result of an impaired cerebrospinal fluid circulation and absorption through a combination of a diffuse inflammatory meningeal response, subarachnoid fibrosis, and blockade of the outlets from the 4th ventricle (Dixon and Heller 1932, Griffith and Roberts 1938, Schurr *et al.* 1953, Granholm 1966, Edvinsson and West 1971). With this procedure a five-fold increase in the VFP was obtained 2 days after the injection (Owman and West 1970). Bilateral superior cervical sympathectomy 4—8 days before the kaolin treatment markedly reduced the degree of intracranial hypertension (Owman and West 1970). In the present investigation *the cerebrospinal fluid circulation was blocked by intracisternally injected kaolin*, and its effect on the VFP was studied by continuous direct recordings in conscious rabbits at various time periods after bilateral superior cervical sympathetic ganglionectomy.

Material and Methods

The material comprised rabbits of either sex, weighing between 2.3 and 3.0 kg, and fed freely with standard pellets (SAN bolagen, Sweden), turnips, and tap water *ad lib.* also during the recording. The animals were divided into the following groups.

Group 1. Six non-sympathectomized control animals. The animals were anesthetized with Nembutal (1.8 ml of a 6 % solution diluted in physiological saline and administered i.v.). Under aseptic conditions a cannula (0.5 mm outer diameter) connected to a syringe via a polyethylene catheter was carefully inserted into the cisterna magna. A total of 0.5 ml of a sterilized kaolin suspension (30 g% hydrated aluminium silicate in 0.9 % physiological saline) [illegible]. The [illegible]

[illegible]

ventricle of the brain. The subsequent recordings of the V[illegible] formed on a Grass Model 7 Polygraph via a Statham Model P23AC transducer as earlier described in detail (Owman and West 1970).

Group 2. Eight animals were injected with kaolin under Nembutal anesthesia as above. Two days later the animals were re-anesthetized with dinitrous oxide-halothan (Fluothane, ICI) inhalation anesthesia and sympathectomy was performed by bilateral removal of the superior cervical ganglia through a small midline incision in the neck. The incision was closed by cutaneous silk sutures. Four to 6 hrs later the above mentioned pressure cannula was inserted for subsequent recordings of the VFP.

[illegible] Four days later the
[illegible] e pressure
[illegible] Group 3,
[illegible] le and the
[illegible] n as zero
reference plane during the recordings

The initial VFP was estimated as the average value obtained for each animal during the first half hour after insertion of the pressure cannula, and the values presented in Fig 1, 2 a, 3 a and 4 a are the means from all animals in the various groups The mean VFP given in the figures at each subsequent 1 hr period was similarly calculated but during a continuous recording during 1 hr

In order to evaluate the pressure changes specifically due to the sympathectomy, the various mean pressure levels given in the curves at each time-period for the non sympathectomized group of animals (Group 1) were subtracted from each corresponding time related mean pressure level in the 3 different groups of sympathectomized animals The resulting *net VFP* thus demonstrates the pressure changes in the sympathectomized groups as related to those in the control group (Fig 2 b 3 b and 4 b)

Results

Group 1 Non-sympathectomized controls Continuous recordings were performed during 44 hours in this group All animals survived and the recording systems remained patent throughout the recording period The initial pressure (*i e* the mean pressure during the first half hour after insertion of the cannula was 70 mm physiological saline (Fig 1) Within the first 7 hrs the mean VFP increased to a maximum of 110 mm saline The pressure then showed a slow progressive decrease to 45 mm saline at the end of the recording period

Group 2 VFP 4—6 hours after sympathectomy Three of the animals in this group died at various periods between 6 and 16 hrs after implantation of the cannula Recordings could be obtained from the remaining 5 animals during a total of 48 hrs

The initial mean pressure was 3 mm saline (Fig 2 a) The VFP then showed a transient increase within 14 hrs with a maximum of approximatively 25 mm saline at 6 hrs Between 14 and 22 hours the VFP was around zero and then it decreased to a level of about −22 mm saline, which remained essentially constant during the remainder of the recording period

Fig 1 Continuous recordings of ventricular fluid pressure (VFP) in 6 non sympathectomized kaolin treated control animals The pressure figure obtained for each time interval for each animal was the mean pressure calculated during a 1 hr period of recording Mean values ——— ± standard error of the mean

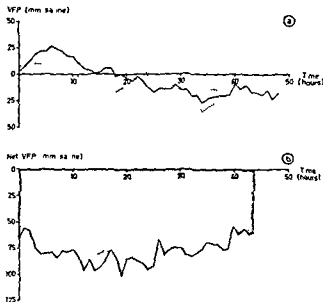


Fig 2 (a) Mean VFP from recordings on 8 kaolin treated animals 4—6 hrs after sympathectomy Mean ——— ± standard error of the mean
(b) Mean net VFP (obtained by subtracting the value at each time interval in the curve of Fig 1 from the corresponding time related values in the curve of Fig 2 a) in kaolin treated animals 4—6 hrs after sympathectomy

The net VFP in this group was very low the pressure was approximately 70 mm below that of the non sympathectomized control animals with a tendency to an increase at the end of the recording period (Fig 2 b)

Group 3 VFP 6 days after sympathectomy The initial mean pressure in this group of animals was 40 mm saline (Fig 3 a) Within 8 hrs after insertion of the cannula the mean VFP rapidly increased to more than 200 mm saline (200 mm was the maximum registration capacity for linearity in the equipment used) After a further 1 hr all animals died

The net VFP calculated as mentioned above started at −30 mm saline (Fig 3 b) and rapidly increased within 9 hrs so that the pressure was more than 125 mm above that of the non sympathectomized control animals

Group 4 VFP 16 days after sympathectomy One of the animals died 14 hrs after starting the recording The recording system remained patent only for 24 hrs in 1 animal and for 35 hrs in another of the remaining animals The recordings from the other 2 animals were discontinued 48 hrs after insertion of the cannula because the VFP fell below the lower sensitivity limit of the recording equipment The initial mean VFP was 95 mm saline (Fig 4 a) The pressure increased within the first 9 hrs to 170 mm This was followed by a rapid continuous decrease down to approximately −75 mm

The net VFP (Fig 4 b) was initially at 25 mm above the pressure in the control animals and then increased to a maximum of 75 mm within 9 hrs Following this the net VFP was variable and decreased down to −120 mm saline

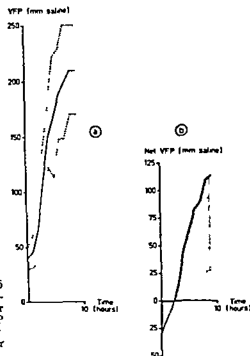
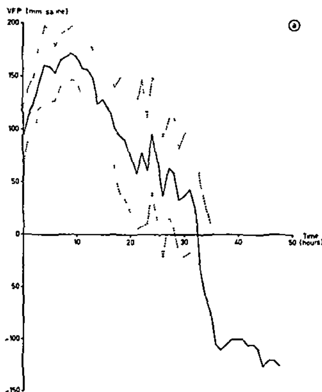


Fig 3 (a) Mean VFP from recordings on 5 kaolin treated animals 6 days after sympathectomy Mean ———, ± standard error of the mean (b) Mean net VFP (curve in Fig 1 subtracted from curve in Fig 3 a) in kaolin treated animals 6 days after sympathectomy

Discussion

Bilateral removal of the superior cervical sympathetic ganglia in animals not treated with kaolin has previously been shown to result in marked changes in the ventricular fluid pressure (VFP) (Edvinsson *et al* 1971 c) Thus, although unaltered shortly after the operation, the VFP is soon reduced by approximately 25 mm saline About 4 days postoperatively, the VFP is 70 mm higher than in unoperated animals it then decreases progressively and 2 weeks after sympathectomy the pressure has returned to essentially the same level as in unoperated control animals These pressure changes have been considered to be related mainly to non-compensated changes (because CSF efflux is probably impaired) in the intracranial blood volume (Edvinsson *et al* 1971 b), the variations being a consequence of an initial noradrenaline leakage (Lundberg 1970, Edvinsson *et al* 1971 b) and disappearance (Malmfors and Sachs 1965, Smith *et al* 1966 Nielsen and Owman 1967, Van Orden *et al* 1967, Edvinsson *et al* 1971 b) from the degenerating sympathetic nerve terminals followed by build-up of denervation supersensitivity of the vascular amine receptors to circulating catecholamines (Langer *et al* 1967, Edvinsson *et al* 1971 b) Principally the same sequence of pressure changes at various time periods after sympathectomy were observed also in the present kaolin treated animals, although the volume pressure levels were markedly exaggerated (see Langfitt *et al* 1965) since CSF absorption was further reduced by the kaolin



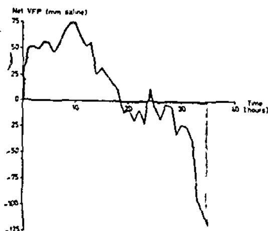


Fig 4 (a) Mean VFP from recordings on 5 kaolin-treated animals 16 days after sympathectomy Mean ——, ± standard error of the mean (b) Mean net VFP (curve in Fig 1 subtracted from curve in Fig 4 a) in kaolin treated animals 16 days after sympathectomy

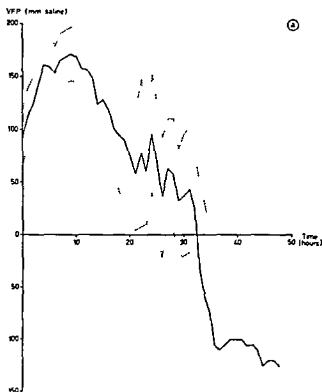
Group I Non-sympathectomized controls In a previous report (Edvinsson *et al* 1971 a) it has been shown that the pressure cannula implanted into the left lateral ventricle of the brain (in animals not treated with kaolin) produces within 10 hrs an increase in the VFP to approximately 70 mm saline This increase in pressure, which was found to be mainly the result of a local, traumatic brain edema, did not subside until 30—35 hrs after implantation probably because of an impaired compensation

lue to reduced CSF absorption It is well known that intracisternally injected kaolin nduces a marked intracranial hypertension As could be expected, pretreatment of the rabbits with kaolin therefore resulted in a further increase in the VFP within 10 hrs by about 40 mm saline 2 days after the treatment The initial VFP after treatment with kaolin was found to be 56 mm saline above that in *animals not receiving kaolin* (Edvinsson and West 1971) This pressure increase is in the same order of magnitude as that obtained in another previous series of experiments in rabbits (Owman and West 1970) The kaolin induced increase in the VFP has been found to show a progressive decline in the course of the recordings, and 24 hours after implantation of the cannula (*i e* 3 days after administration of the kaolin) the VFP had returned to approximately the same level as in untreated animals (Edvinsson and West 1971) It is conceivable that this normalization of the VFP in kaolin treated animals reflects an increased cerebrospinal fluid absorption via normal pathways not involved in the kaolin blockade (Bering and Sato 1963, Sahar *et al* 1969 a), via the formation of alternate pathways for its absorption (Sahar *et al* 1969 a and b, 1970), and/or a reduced cerebrospinal fluid formation (Heisey *et al* 1962 Calhoun *et al* 1967, Hochwald *et al* 1969, Lorenzo *et al* 1970)

Group 2 4—6 hrs after sympathectomy Three of the animals in this group died at the beginning of the recording period The VFP in these animals, however, were in the same order of magnitude as the pressures in the surviving animals and they have thus not to any greater degree influenced on the principal pattern of the mean pressure curve obtained in the group Thus the animals did probably not die from an exceptionally high VFP but perhaps rather as a consequence of an impaired general condition

The low net VFP (*i e the total mean VFP minus the corresponding time related* pressure values in the non sympathectomized controls) can be explained in the following way Blockade of the cerebrospinal fluid circulation with kaolin has induced intracranial hypertension and in order to compensate the increased VFP, the above mentioned changes in formation and absorption occur As a consequence of this the VFP will be lowered (Edvinsson and West 1971) In addition the sympathetic ganglionectomy causes the noradrenaline transmitter to leak from the degenerating nerve terminals (Lundberg 1970) thus constricting the brain vessels (Sokoloff 1959 Rosenblum 1965 Edvinsson *et al* 1971 b), which reduces the intracranial blood volume These two different events and the suggested decline in traumatic brain edema (see West 1971) at this postoperative stage will act together and result in a low net VFP

Group 3 6 days after sympathectomy Six days after sympathectomy the noradrenaline has disappeared from the degenerating constrictor nerves to the brain vessels (Sokoloff 1959, Malmfors and Sachs 1965 Smith *et al* 1966 Nielsen and Owman 1967) The vessels are therefore expected to dilate (Edvinsson *et al* 1971 b) resulting in an increase in intracranial blood volume This will add to the hypertension induced by kaolin injection 2 days earlier However, already at this stage the



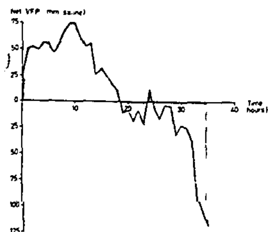


Fig 4 (a) Mean VFP from recordings on 5 kaolin treated animals 16 days after sympathectomy Mean ——— ± standard error of the mean (b) Mean net VFP (curve in Fig 1 subtracted from curve in Fig 4 a) in kaolin treated animals 16 days after sympathectomy

Group 1 Non sympathectomized controls In a previous report (Edvinsson *et al* 1971 a) it has been shown that the pressure cannula implanted into the left lateral ventricle of the brain (in animals not treated with kaolin) produces within 10 hrs an increase in the VFP to approximately 70 mm saline This increase in pressure, which was found to be mainly the result of a local, traumatic brain edema, did not subside until 30—35 hrs after implantation probably because of an impaired compensation

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Nervous Influence on the Pulmonary Capacitance Vessels in the Rat

By

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Abstract

AARSETH, P *Nervous influence on the pulmonary capacitance vessels in the* :
Acta physiol scand 1971 83 60—69

It has previously been shown in experiments on rats that the pulmonary capacitance vessels play an important role as a blood depot in this species In the present experiments one has attempted to evaluate the vasomotor nervous influence on this depot Pulmonary blood volume was estimated from measurements of [125]I tagged albumin and [51]Cr tagged erythrocytes in the excised lungs of rats which had been rapidly frozen in liquid nitrogen Partial pulmonary denervation was achieved beforehand in two groups of animals by cutting the left thoracic vagosympathetic nerve trunk Animals from one of these groups were frozen without further treatment whereas those from the other group were exposed to a standardized blood loss immediately before being immersed in liqu
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than did their right lung Furthermore
increased in animals with such partly
volume observed subsequent to a blood loss was less marked in animals where the left vagosympathetic nerve trunk had been cut than in animals with intact pulmonary innervation It is concluded that the pulmonary blood volume in the rat is influenced through vasomotor nerves both when a blood loss has taken place and during normal circulatory conditions

It has been shown that the pulmonary blood volume in rats is reduced by as much as 25 % just after a general blood loss corresponding to 12 % of the animals total blood volume (Aarseth 1970 a) Such a large relative volume reduction in one vascular compartment reveals what might be called a depot function of that vasculature It is not clear, however if and to what extent the pulmonary blood volume is nervously controlled Experiments on isolated lungs indicate that the pulmonary sympathetic innervation may markedly modulate blood volume in this vascular territory *It has thus been shown in non perfused dog lung lobes at high intratracheal* pressures that the intravascular volume is diminished on both the arterial and venous side of the compressed capillaries when the vagosympathetic nerve is stimulated (Daly and Waaler 1961 Daly Ramsay and Waaler 1970) During perfusion of such a preparation, nerve stimulation was shown to cause a considerable blood volume reduction (Daly *et al* 1970) Using a similar preparation Aarseth, Nicolaysen and

Waaler (1971) demonstrated that the weight of the lungs was markedly reduced when adrenergic nerves to the organ were stimulated. The weight fall, which was thought to reflect a blood volume reduction, corresponded to about 5—10 % of the total blood volume in the preparation. Szidon and Fishman (1969) also reported that the volume of the pulmonary vascular bed could be reduced upon stimulation of sympathetic nerves to the lung. It must be assumed therefore that the pulmonary vessels are supplied with nerve fibres through which it would be possible to exert some control over the pulmonary blood volume. However, it has not been convincingly demonstrated that such a nervous control is of any importance in an intact organism. In experiments on dogs Chien and Usami (1969) attempted to analyze this problem. They exposed animals to standardized blood losses and compared the effects seen in totally sympathectomized animals with those seen in control animals. The pulmonary blood volume reduction upon a blood loss was found to be the same in the two groups. These authors concluded therefore that the post hemorrhagic blood mobilization from the pulmonary vascular bed was merely the result of passive vascular recoil, following changes in intravascular pressures. However, this conclusion can probably be questioned since total sympathectomy will markedly affect the whole cardiovascular system with all its vascular beds.

The aim of the present experiments has been to get more information on the role of the pulmonary vascular innervation for the control of lung blood volume. The method of rapidly freezing differently treated whole rats (Everett, Simmons and Lasher 1956, Aarseth 1970 a) has been used. The results indicate that the post-hemorrhagic mobilization of blood from the pulmonary vasculature is partly depending on an intact pulmonary innervation. A preliminary report of the results achieved has been given before (Aarseth 1970 b).

Methods

Inbred male Wistar spf rats weighing 190—260 g were used. The animals had free access to a standard pellet food and to water until the experiments started.

Surgical procedures, lung denervation. 34 animals in one group were anesthetized by an i.p. injection of 40 mg/kg b.w. of pentobarbitone (Nembutal®, Abbott diluted 1 to 4 in isotonic saline). Tracheostomy was carried out and ventilation performed with room air at intermittent positive pressure and at a frequency of 36/min. The peak inflation pressure and the end expiratory pressure were +10—12 cm and +2 cm H_2O respectively. In 3 preliminary experiments an appropriate degree of ventilation was chosen under the guidance of several blood gas anal [illegible]

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The methods for *blood volume estimation* and for the application of an acute blood loss have previously been presented in detail (Aarseth 1970 a) and will only be briefly described here. The animals were anesthetized with 30 mg/kg of pentobarbitone i.p. Through a catheter *in the right femoral vein heparin (0.2 ml containing 800 I.U.* ...
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groups w... ...e right and left lungs were later removed from the frozen animals and placed in separate tubes. A standardized sample was also taken from a muscle groups in the left thigh. All tissue samples were weighed. Hematocrit was measured in the blood samples taken using a microhematocrit centrifuge. From the count rates for ^{125}I and ^{51}Cr in the injected isotope mixture, in the shed blood and in the tissue samples, total pre-bleeding blood volume, pulmonary blood volume and muscle blood content were calculated (Aarseth 1970 a). The weight of the lung tissue proper was calculated by subtracting the weight of the pulmonary blood from the total weight of the lungs.

"Pulmonary hematocrit" was defined as pulmonary erythrocyte volume/(pulmonary plasma volume + pulmonary erythrocyte volume).

Results

The effect of a blood loss on the pulmonary blood volume in sham-operated rats. In the present series of experiments the left thoracic vago-sympathetic nerve trunk was cut in order to see if this would influence the depot-function of the pulmonary vascular bed. *This procedure involves major surgery. It was necessary therefore to evaluate* the effect which the thoracotomy *per se* might have upon the post-hemorrhagic mobilization of blood from the pulmonary vascular bed. The 10 sham-operated animals were exposed to a standardized blood loss (mean value corresponding to 12.6 % of total blood volume) 4 days after thoracotomy, and their pulmonary blood volume estimated immediately thereafter. The results are shown in Table I. For comparison pulmonary blood volume in another group of bled animals is also shown.

TABLE I. Comparison of the pulmonary blood volumes after a standardized blood loss in *thoracotomized and non-thoracotomized animals. The left-sided thoracotomy was* performed 4 days before exposure to the blood loss. Total pulmonary blood volumes as well as separate values for the left and the right lungs are given for both groups of animals. Mean values for body weight, total blood volume and percental blood loss are also given.

Treatment	n	Body weight (g)	Total blood volume (ml) (before blood loss)	Blood loss (%)	Pulmonary blood volume µl	Left lung blood volume (µl	Right lung blood volume (µl
Thoracotomy, bleeding	10	224	14.05	12.6	1279	586	692
Bleeding*	14	242	14.08	11.9	1385	639	746

* Data from a series of experiments published earlier. Aarseth 1970 a.

TABLE II Pulmonary blood volume in non bled animals in which the left vago-sympathetic nerve trunk had been cut, compared to pulmonary blood volume in non treated non bled control animals Wilcoxon s two-sample test has been used for calculations of p

Characterization of animal groups	n	Body weight (g)	Total blood volume (ml)	Lung blood volume (μl)	Lung blood volume in % of total blood volume	Left lung blood volume (μl)	Right lung blood volume (μl)	Ratio between blood volumes in left and in right lung	Muscle blood content (μl g)
Thoracotomy, Left-sided denervation	19	212	12 78	2045	16 5	1004	1118	0 904	23 2
Control group* No treatment	17	244	14 08	1845	13 2	832	1013	0 821	20 9
Difference				200	3 3	172	105	0 083	
				p=0 195	p=0 023	p=0 068	p=0 33	p=0 059	

* Data from series of experiments published earlier (Aarseth 1970a)

The mean blood loss in these other animals, which had not been thoracotomized, was 11 9 % (Aarseth 1970 a) There are very small differences between the pul monary blood volume values found in the two groups

Pulmonary blood volume in animals where the left vagosympathetic nerve trunk had been cut One would expect lung blood volume to alter on denervation of the organ if the pulmonary capacitance vessels are under some sort of continuous nervous influence. In the present investigation pulmonary blood volume was estimated subsequent to the severence of the left vago-sympathetic nerve trunk in order to evaluate such a possibility Table II shows a comparison between pulmonary blood volumes found in such a group with partial denervation and pulmonary blood volumes found n a previously investigated group of animals with intact innervation (Aarseth 1970 a) The two groups happened to have somewhat different mean total blood volumes and their pulmonary blood volume values can therefore not be compared directly (Aarseth 1970 a) Instead pulmonary blood volumes expressed as per cent of the total blood volumes have been compared Pulmonary blood volume corresponded to as much as 16 5 % of the total blood volume in the animals in which the left vago-sympathetic nerve trunk had been cut In the group with intact inner vation only 13 2 % of the total blood volume was found within the lungs This dif ference in relative pulmonary blood volume is significant at a 2 3 % level It can also be seen from Table II that the ratio between blood volume in the left and the right lung was greater in the denervated group (0 904) than in the control group (0 821) The significance level of this difference is 5 9 % The results indicate that a volume increment of the pulmonary capacitance vessels takes place when the left vago-sympathetic nerve trunk is cut This increment seems to be most marked in the ipsilateral lung

TABLE III [illegible]

two sample test has been used for calculation of p

Characterization of animal groups	n	Values from pre *bleeding* situation		Values found or calculated in post bleeding situation					
		Body weight (g)	Total blood volume (ml)	Lung blood volume (μl)	Lung blood volume in % of *total* blood volume after bleeding	Left lung blood volume (μl)	Right lung blood volume (μl)	Ratio between blood volume *in left and in right* lung	Muscle blood content (μl g)
Thoracotomy, Left sided denervation, Bleeding	15	202	12 60	1752	15 9	815	936	0 880	19 5
Thoracotomy, Bleeding	10	224	14 05	1279	9 9	586	692	0 836	20 6
Difference				473 $p = 0\ 012$	6 0 p 0 002	229 p 0 008	244 $p = 0\ 014$	0 044 $p = 0\ 063$	

The effect of a blood loss on the pulmonary blood volume in animals where the left vago sympathetic nerve trunk had been cut Another group of 15 animals where the left vago-sympathetic nerve trunk had been cut, were exposed to a standardized blood loss (with mean value corresponding to 12 7 % of the total blood volume) and their pulmonary blood volumes estimated immediately thereafter In Table III the values found in this group are compared with the values found in 10 sham operated animals which had suffered a similar blood loss The animals with the vago-sympathetic nerve trunk cut had a much greater fraction of their total remaining blood volume in the pulmonary vascular bed than had the animals with *intact innervation The mean values here were 15 9 % and 9 9 % respectively* ($p =$ 0 002 The denervated animals had a smaller total blood volume but at the same *time a larger lung blood volume than had the sham-operated animals* The pulmonary blood volume differences were most marked for the left lungs The ratio between the blood volume in the left lung and in the right lung was thus 0 880 in the 'denervated group and 0 836 in the sham-operated group ($p = 0\ 063$

Post bleeding mobilization of blood from lungs and from skeletal muscle in rats in which the left vago sympathetic nerve trunk had been out Animals in which the left vago-sympathetic nerve trunk had been cut and which were exposed to a standardized blood loss had a mean post bleeding pulmonary blood volume which was 14 3 % smaller ($p = 0\ 077$) than the mean value found in denervated but nonbled animals (Fig 1) In rats with intact pulmonary innervation a similar blood loss

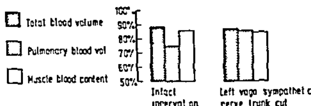


Fig 1 Mean reductions in total blood volume in pulmonary blood volume and in skeletal muscle blood content in two groups of animals The animals of one group were previously thoracotomized and their left vago-sympathetic nerve trunk cut The animals in the other group had intact pulmonary innervation The values for both groups are given as levels relative to those in similarly treated non bled groups

resulted in a reduction of pulmonary blood volume by as much as 25 % Skeletal muscle blood content was slightly more reduced in the bled animals with partly denervated lungs than in the bled animals with intact pulmonary innervation (16 % as against 14 % reduction respectively (Fig 1))

The blood volume in the total mass of skeletal muscle can be estimated from the blood content found in the muscle samples taken if one assumes that all the skeletal muscles correspond to about 45 % of b w (Caster *et al* 1956) Thus the approximate extent of blood mobilization from the lungs and from the muscle can be calculated by subtracting the appropriate values found in bled animals from the values found in the corresponding non bled animals Results from such calculations are shown in Fig 2 In the bled animals with intact pulmonary vascular innervation more blood was mobilized from the lungs than from the total mass of skeletal muscle However in animals in which the left vago-sympathetic nerve trunk had been cut somewhat less blood was contributed from the lungs than from the skeletal muscles

Pulmonary hematocrit" and weight of lung tissue For unknown reasons the pulmonary hematocrit in rats was found to increase subsequent to a bleeding (Aarseth 1970 a) Such an increase was seen also in the sham-operated bled animals in the present series of experiments Animals in which the left vago-sympathetic nerve trunk had been cut appeared to have a somewhat lower pulmonary hematocrit and there was no significant difference between the values found in bled and in non bled animals (Fig 3)

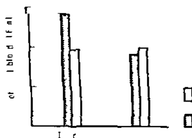


Fig. 2. Comparison of calculated amount of blood mobilized from the lungs and from the skeletal muscle subsequent to a standardized bleeding in two groups of animals The animals in the first group had an intact pulmonary innervation The animals in the other group had been thoracotomized and their left vago-sympathetic nerve trunk cut The blood loss was nearly the same in the two groups 11 9 % and 12 7 % of the total blood volume respectively



Fig 3 Pulmonary hematocrit and weight of lung tissue proper in 5 differently treated groups of animals The data in the groups with no treatment are taken from a series of experiments published earlier (Aarseth 1970 a)

The weight of the lung tissue proper has previously been found to be somewhat reduced after a 12 % bleeding (Aarseth 1970 a) This reduction was more marked the larger the blood loss had been and was interpreted as being due to inward transvascular flux of fluid, probably as a result of a lowered capillary pressure In the present series of experiments such a post bleeding reduction in lung tissue weight was observed neither in the sham operated nor in the denervated animals On the contrary unbled animals had a markedly lower lung tissue weight than had the bled animals ($p = 0.001$) Fig 3)

Discussion

The method used for blood volume estimations have been discussed earlier (Aarseth 1970 a) It was concluded then that the anesthesia employed did not abolish the vascular reflexes which maintain the mean blood pressure after a blood loss of about 12 % In the present investigation the animals had to be thoracotomized 4 days previous to the blood volume estimations In the 4 days between the thoracotomy and the bleeding the animals did show a mean weight loss of about 5 % and their hematocrit was slightly increased as compared to the value in animals which had not been operated However the mean pulmonary blood volume after a blood loss was the same in sham-operated as in non operated animals (Table I) It appeared therefore that the performance of thoractomy did not *per se* influence the observed depot function of the pulmonary vascular bed

The lung receives vasomotor nerve fibres of different types and from several sources (Daly and Hebb 1966) There is more knowledge available about this innervation in the dog than in other animal species However the pattern of innervation appears to be much the same also in other species (Daly and Hebb 1966, Greene 1963) According to Daly and Hebb the dog lung receives parasympathetic vasdilator fibres which have their origin in the vagal nuclei Sympathetic vasomotor fibres to the lung relay in the lower cervical and upper thoracic sympathetic ganglion. These fibres, which are predominantly vasoconstrictor ones join the vagus in the

upper part of the thorax and run peripherally in the thoracic vago-sympathetic trunk By cutting the left thoracal vago-sympathetic trunk one should therefore destroy all the pulmonary sympathetic nerve fibres running on the left side

Anatomical studies (Mollgaard 1912) and physiological studies in dogs (Daly and Hebb 1954) have demonstrated that there is some crossed parasympathetic as well as sympathetic innervation to the lungs Daly and Hebb (1954) used chronically pneumectomized dogs and measured changes in pulmonary arterial pressure under constant volume perfusion conditions They found pulmonary vasomotor responses to occur on both ipsilateral and contralateral vago-sympathetic nerve stimulation The responses seemed to be of greater magnitude when the ipsilateral nerve trunk was stimulated In the present investigation the severance of pulmonary nerves similarly appeared to have the most marked effect on the ipsilateral pulmonary capacitance vessels In both bled and non bled animals where the left vagosympathetic nerve had been cut did the left lung contain relatively more blood than the right one (Table II and III)

Theoretically it would have been preferable in this type of experiments to denervate the pulmonary vasculature completely This could, however, not be achieved for technical reasons It would hardly be possible to carry out separate cutting of all nerve fibres to both lungs—and of such fibres only One had to use cutting of the whole vago-sympathetic trunk therefore The cutting of this trunk on both sides would have left the heart completely denervated This had to be avoided as such denervation would most probably markedly affect the more general circulatory responses to a marked blood loss Furthermore such a procedure would have necessitated much more drastic surgery with opening of the chest on both sides

One sided cutting of the vago-sympathetic trunk was employed therefore and thereby only one half of the pulmonary vascular innervation was severed and probably more fibres to the left than to the right lung Cutting of the left-sided vago-sympathetic trunk does not represent selective pulmonary denervation Fibres to other organs *e g* to the heart will also be severed by cutting this nerve trunk The cutting of such fibres on the left side might therefore conceivably affect the animals cardiac response to a 12 % blood loss even if the innervation on the right side is intact. General circulatory alterations of this and other types should however influence the vascular beds in both lungs to the same extent One would therefore not expect such alterations to cause the observed changes in the blood volume ratio between the two lungs (Table II and III) It appears therefore that the changes seen in lung blood volume subsequent to the cutting of the left vago-sympathetic nerve trunk must be related to the severing of pulmonary nerve fibres

Another problem related to the denervation technique used is the development of denervation supersensitivity Such supersensitivity seems to develop in all denervated structures probably olso to some extent in the lungs when the one vago-sympathetic nerve trunk is cut If denervation supersensitivity develops then the pulmonary vascular bed would become more sensitive towards circulating catecholamines which are probably being released at an increased rate during the bleeding period

before the animals are frozen However, both adrenaline and nor adrenaline have been shown to reduce the blood volume of isolated perfused lungs (Hauge, Lunde and Waaler 1967) Any supersensitivity would thus, if present, presumeably make the effect of sympathetic denervation less evident

In the cat submandibular gland Emmelin and Muren (1952) found that some supersensitivity could be observed on the third day after denervation, but it did not reach its maximum level until 8—10 days had elapsed Axelsson and Thesleff (1957) found the same time course for the development of supersensitivity in denervated striated cat muscle A 4 days waiting period between thoracotomy with denervation and further experimentation thus seemed to combine the demands for a relatively good recovery after the operation and that of a small denervation supersensitivity

The denervation procedure employed did affect the pulmonary blood content and the mobilization of blood from the pulmonary vasculature to a considerable extent (Fig 1 and 2, Table II and III) in spite of the fact that some supersensitivity might have developed, and even if only one half of the pulmonary vasomotor fibres were cut. Firstly, animals in which the left vago sympathetic nerve trunk had been cut had a larger of their total blood volume in the pulmonary vascular bed than had the animals with intact pulmonary innervation Secondly the left lung of the animals with left sided denervation contained relatively more blood than in the control animals Finally the blood mobilization from the pulmonary vascular bed subsequent to a bleeding was smaller in the animals with partly denervated lungs than in animals with intact innevation, and there seemed to be a compensating increase in blood mobilization from the skeletal muscles in the former group The data in the present series of experiments do not allow accurate estimations of how much of the post hemorrhagic blood mobilization is due to vasomotor nervous activity and how much to passive recoil of the vessels Still the findings presented makes it difficult to escape the conclusion that pulmonary vasomotor innervation influences the volume of the capacitance vessels in the organ both in the normovolemic and in the hypovolemic situations

The values found for pulmonary hematocrit and for lung tissue weight (Fig 3) are somewhat difficult to interpret Animals in which the left vago sympathetic nerve trunk had been cut appeared to have a lower pulmonary hematocrit than has previously been found in animals with intact innervation (Fig 3) Also whereas in animals with intact pulmonary innervation lung hematocrit increased markedly after a blood loss this was much less marked in the animals with one sided denervation Furthermore in the animals with one sided denervation the weight of the lung tissue proper which was remarkably low showed a marked increase subsequent to a blood loss This is contrary to what is found in animals with intact pulmonary innervation (Aarseth 1970 a) Possibly the denervation procedure does in some way disturb the ratio between pre and post-capillary resistance in the organ with reduced capillary pressure as a consequence In this connection it is of interest to note that in the rat lung Hebb (1969 could find adrenergic nerve fibres only to the intra

pulmonary veins and not to the intrapulmonary arteries It is possible that the low lung hematocrit seen in the animals with the left vago-sympathetic nerve trunk cut may also be a result of changes in their pulmonary micro-circulation

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ATP Hydrolysis and Noradrenaline Transport in Purified Vesicles from Splenic Nerve Trunk

By

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Abstract

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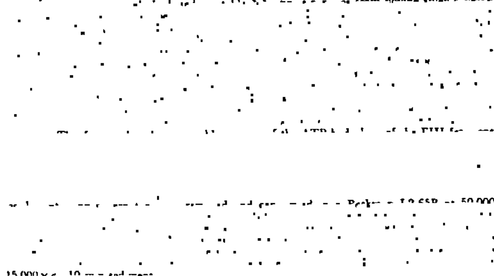
A [illegible] ner [illegible] of [illegible] ly [illegible] tions of Na^+ and K^+ salts had little effect. Concentrations of NA and dopamine between 0 1 and 10 0 μg/ml had no effect A concentration of 5×10^{-8} M reserpine, which produced 30—50 % inhibition of NA exchangeability and influx rate, had no effect on ATP hydrolysis under a wide variety of conditions This concentration of drug causes no obvious evidence of vesicle damage at the electron microscopic level The data do not support the possibility of a link between ATP hydrolysis and NA transport in either direction across the membrane of the isolated nerve trunk vesicle

A recent advancement in the purification of noradrenaline (NA) storage vesicles isolated from bovine splenic nerve trunk has been described (Lagercrantz, Klein and Stjarne 1970) The NA protein ratio in the purest fraction (FIII) averaged 3 44 μg NA/mg protein, a several fold increase over values previously reported Morphological evidence for vesicle purity has also been presented (Thureson-Klein *et al* 1970, Klein and Thureson-Klein 1971) It is of interest to determine the capability of this vesicle preparation to hydrolyse adenosine triphosphate (ATP) and to evaluate any possible relationship of the enzyme to catecholamine transport across the vesicle membrane.

The present communication includes data on the ATP hydrolytic activity of the various gradient fractions It describes a simple procedure for further purification of the NA vesicle fraction The latter has been tested for ATP hydrolytic activity as affected by Mg^{++}, Ca^{++}, Na^+, K^+, NA, dopamine (DA), reserpine and chelators The data are compared with known effects on NA exchange in vesicle preparations under similar conditions

Methods

urther purification of isolated NA storage vesicles Methods for producing isolated vesicle



...icle suspensions were diluted so that the amount of ... (0—20 %) of the total substrate available A total of ... was used and the enzymatically released inorganic ... Sen 1967) A 5 to 10 min period was allowed for ... prior to initiating the hydrolysis by the addition of ATP Reactions were allowed to proceed for 30 min, during which time they remained linear, at 37—38° C in an agitating water bath Reactions were stopped by mixing in 0.5 ml of perchloric acid reagent and the tubes placed immediately in an ice bath. Any precipitated protein was sedimented to avoid possible interaction when the molybdate reagent was subsequently added to aliquots ... gs at 740 mμ were b... ...thod as employed w... ...was prepared as the ... The Lowry method ...

Results and Discussion

A test of the method ATP hydrolysis by isolated bovine adrenal medullary vesicles Measurements of ATP hydrolysis by relatively pure preparations of bovine adrenal medullary vesicles have been made in several laboratories In order to test the comparability of the present method with values given in the literature, 3 expts using sucrose step-density gradients were performed to produce isolated vesicle preparations from bovine adrenal medulla The purified fraction contained ~ 300 μg sedimentable catecholamine/mg protein Under the same conditions used for isolated nerve trunk vesicles (legend Fig 1), the specific activity (SA) of the Mg^{++}-activated ATP hydrolysis was 0.06 μM P_i/mg protein/min Addition of various combinations of Na^+ and K^+ salts produced only small increases in hydrolysis rate Ca^{++} activation produced less hydrolysis than Mg^{++} Addition of Na^+ and K^+ salts resulted in inhibition of Ca^{++}-activated ATP hydrolysis The SA and relative effects of activators agree well with those of others (Banks 1965, Taugner and Hasselbach 1966, 1968)

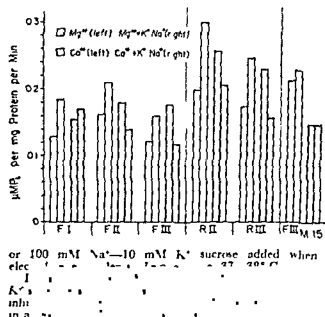


Fig 1 Open bars ATP hydrolysis with Mg^{++} (to left) and $Mg^{++}+K^{+}-Na^{+}$ (to right) Shaded bars ATP hydrolysis Ca^{++} (to left) and $Ca^{++}+K^{+}-Na^{+}$ (to right) FI FII FIII = original sucrose heavy water continuous density gradient fractions in order of increasing density, means of 6 expts RII RIII = FII plus FIII subjected to a second identical density gradient separation means of 5 expts $FIII_{M\ 15}$ = highest sedimentable NA protein fraction (see Methods and text), means of 3 expts Final concentrations 20 mM tris HCl buffer at pH 7.2 5 mM tris HCl buffer at pH 7.2 mM Mg^{++} or Ca^{++} 5 mM tris ATP either 100 mM K^{+}—10 mM Na^{+} or 100 mM Na^{+}—10 mM K^{+} sucrose added when needed to maintain ~0.310 M in elec ... 37 ... 38° C

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ATP hydrolyses by the original sucrose heavy water density gradient fractions FI, FII, FIII It was previously found (Lagercrantz, Klein and Stjärne 1970) that FI contained most of the glucose-6-phosphatase (microsomic), thiamine pyrophosphatase (Golgi membrane) and acid phosphatase (lysosome) activity when compared to the higher NA containing fractions FII and FIII In fact, FIII had no demonstrable microsomal enzyme activity while having the highest average sedimentable NA protein ratio In contrast after removal of at least half of the CyOx activity by an initial 10 500 × g 10 min fractional centrifugation of the nerve trunk homogenate the remaining CyOx was distributed about equally (12 18 %) among the 3 fractions Although the total mitochondrial enzyme was greatly reduced from the original homogenate the SA of CyOx increased about 3 times in FIII

It can be seen in Fig 1 that the ATP hydrolyses activated by Mg and Mg^{++}+ K—Na differ very little in the 3 fractions The stimulation by various combinations of K and Na salts ranges between 20 and 42 % Note that the SA values do not indicate distribution of the total enzyme activity This is much greater in FI in agreement with the results of others (Hortnagl Hortnagl and Winkler 1969) Activation by Ca and Ca + K Na do show differences The SA of Ca activated ATP hydrolysis is similar in all three fractions but further addition of various combinations of K and Na salts tends to stimulate slightly in FI while producing significant inhibition in FII and FIII

ATP hydrolyses by repurified fractions RII and RIII It was reported previously (Lagercrantz, Klein and Stjärne 1970) that in some experiments the original fractions FII and FIII were removed diluted to isotonicity and then subjected to a

second identical sucrose heavy water density gradient fractionation giving rise to fractions RII and RIII This resulted in a further decrease of the 7 % glucose 6-phosphatase activity remaining in FII and in acid phosphatase activity of FII and FIII, both activities being decreased to about half The CyOx activity was also reduced to less than half in RII but it was usually increased in RIII (unpublished data) Thus there was little net loss in SA of CyOx in RII + RIII compared to the original FII + FIII The NA protein ratio of RII was at least doubled approaching that in the original FIII However this ratio was little changed in RIII and at the expense of about 50 % less vesicle yield Thus the time consuming repurification was of no practical advantage in terms of total yield or improved overall NA protein ratios

In 5 expts repurified fractions RII and RIII were tested for ATP hydrolytic activity (Fig 1) There is little difference in the qualitative effects of the various cations compared to those in the original FII and FIII The SA is increased by repurification indicating that some selective removal of non ATP hydrolysing protein had occurred

ATP hydrolysis by fraction $FIII_M$ subjected to additional purification The fraction $FIII_M$ is produced by a modification of the sucrose heavy water density gradient which results in the recovery of FIII plus part of FII in a much condensed band (Klein and Lagercrantz 1971 also see Methods) In 3 expts the $FIII_M$ was diluted to isotonicity and fractionally centrifuged at 15 000 × g for 10 min Up to 86 % of the remaining CyOx activity was removed while retaining 50—60 % of the sedimentable NA in the supernatant The vesicles harvested from the supernatant had increased NA protein ratios up to two times the original $FIII_M$

The ATP hydrolytic activity of fraction $FIII_{M\ 15}$ is shown in Fig 1 and Table I Three major changes occur in the characteristics of ATP hydrolysis The basic Ca activated ATP hydrolysis for the first time falls to values significantly lower than those with Mg It is also no longer inhibited by addition of various combinations of K and Na salts The basic Mg^{++} activated hydrolysis is no longer significantly stimulated by the addition of various combinations of K and Na salts To the extent comparable these findings agree with data of others on isolated splenic nerve trunk vesicles (Burger Philippu and Schumann 1969) and on isolated adrenal medullary vesicles (Banks 1965) One can conclude that in the original fraction FIII and in repurified RIII the remaining mitochondrial contamination was largely responsible for the observed K—Na stimulation of Mg and inhibition of Ca^{++} activated ATP hydrolyses

Effects of noradrenaline dopamine reserpine and chelation on ATP hydrolysis by $FIII_{M\ 15}$ The purest NA containing fraction $FIII_{M\ 15}$ was tested for ATP hydrolytic activity under conditions similar to those known to enhance and inhibit NA exchange in the vesicles (Table I) When all traces of Mg and Ca were removed from the medium by 5 mM EDTA no ATP hydrolysis occurred The ATP hydrolysis in the presence of 5 mM Mg^{++} was 0 22 and with Ca was 0 15 μM P_i/mg protein/

TABLE I

Temperature, 37—38° C

Basic Medium	Micromoles Inorganic Phosphate per mg Protein per Minute						
			Other Additions				
		% Δ	0 1—10 0 μg NA/ml	0 1—10 0 μg DA/ml	5×10⁻⁸ M Reserpine	NA + Reserpine	DA + Reserpine
Mg	0 214	—	0 212	0 217	0 218		
Mg +EGTA	0 141	—34					
Mg+K-Na	0 230	+7	0 227	0 227	0 235	0 229	0 230
Mg+K-Na +EGTA	0 153	—29	0 155	0 157		0 144	0 149
Ca	0 149	—	0 151	0 147	0 145	0 149	0 135
Ca+K-Na	0 149	0	0 152	0 138	0 149	0 152	0 154
EDTA	0						

min The addition of 5 mM EGTA (Ca^{++} chelation) to any of the various media containing Mg^{++} produced a decrease in the rate of ATP hydrolysis about 30 %. This suggests that traces of Ca^{++} are required for a portion of the Mg^{++}-activated ATP hydrolysis

The addition of 0 1 to 10 0 μg NA/ml to any of the media had no effect This range includes lower concentrations in which Mg-ATP is particularly effective in preventing net loss of NA from the vesicles or in promoting net uptake of NA in partially depleted vesicles (Euler and Lishajko 1967 a, b) The higher end of this range also inhibits net loss of NA, but this is less dependent on a specific requirement for Mg ATP as there is also a large increase in the passive inward driving force for NA Dopamine in a similar concentration range as NA has no effect

At 5×10^{-8} M reserpine produces no effect on ATP hydrolysis in the variety of media tested This concentration of reserpine was chosen because in recent unidirectional isotope permeability studies (Klein and Lagercrantz 1971) it was found to cause a reversible 30 to 50 % decrease of the usual 100 % NA exchangeability without drug The influx transfer coefficient was reduced 25 to 30 % with lesser effects on efflux This occurred in an isotonic medium containing 5 mM Mg-ATP, 0 5 μg l-NA/ml and 20 mM tris-HCl buffer at pH 7 2—7 4 and 30° C A concentration of 2×10^{-7} M reserpine completely inhibited NA exchange for all practical purposes (Euler and Lishajko 1967 b Klein and Lagercrantz 1971) These drug con

centrations do not accelerate the loss of NA from vesicles compared to identical samples without drug In fact, 10^{-7} M reserpine acts as if it "corks up" the vesicle membrane to NA flow in both directions, and will actually retard the slow loss of vesicle NA which occurs even with Mg-ATP present

Using an isolated fraction of NA vesicles from bovine splenic nerve trunk, the results of others differ in some respects (Burger, Philippu and Schümann 1969) They found that a very high concentration of reserpine, 6×10^{-5} M, produced decreases in Mg^{++}-activated ATP hydrolysis of 26 % and of Ca^{++}-activated hydrolysis of 23 % There was also a net loss of vesicle NA of 45 % and 5 %, respectively, after loading the vesicles in a medium containing Mg ATP and relatively high concentration of NA (10 0 μg/ml) They did not give corresponding data on NA fluxes or exchangeability In our experience, reserpine in this high concentration range produces irreversible biochemical and ultrastructural damage to the vesicles resulting in membrane rupture (unpublished observations) This finding is strongly supported by other recent work (Palm, Grobecker and Bak 1970) Reserpine at 5×10^{-8} M produces no electron microscopic evidence of a deleterious effect on the nerve trunk vesicles when compared to Mg ATP incubated preparations without drug (Klein and Lagercrantz 1971) It has been shown that competitive inhibition between reserpine and NA exists over a considerable concentration range (Stjärne 1964, Klein and Lagercrantz 1971) The membrane stabilizing effects of reserpine in the more 'therapeutic' range of 10^{-8} to 10^{-7} M can be reversed by raising the NA concentration in the medium from 0 5 μg/ml to 1—3 μg/ml (Klein and Lagercrantz 1971) The fact that high concentrations of NA (10 0 μg/ml) were used in the Mg ATP medium by others (Burger, Philippu and Schumann 1969) may have necessitated the need for a high concentration of reserpine to demonstrate an inhibitory effect

The present results indicate that a concentration of reserpine, which reversibly produces nearly half maximal inhibition of vesicle NA exchangeability and influx produces no effects on ATP hydrolysis under a wide variety of conditions (NA concentration cation activation and chelators) Also the possible substrates for transport, NA and DA produce no effects on ATP hydrolysis under the same variety of conditions On this basis the authors are obliged to conclude that the data do not support the possibility of a link between ATP hydrolysis and NA transport in either direction across the membrane of isolated splenic nerve trunk vesicles It does support the presence of one or more enzymes capable of Mg^{++}- and Ca^{++} activated ATP hydrolyses As the enzyme does not appear to require any combination of K^+ and Na^+ salts for maximal activity one can also conclude that it is not of the typical ouabain sensitive variety In support of this is the lack of effect of high concentrations of ouabain on NA exchange in the vesicles (Euler and Lishajko 1965)

... from the National ... e Swedish ... Professor

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Studies on the Site and Mechanism of Action of the Growth Inhibiting Effects of Estrogens

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Abstract

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The effect of estrogens and growth hormone on the incorporation rate of sulfate into costal cartilage of the mouse was studied *in vivo* and *in vitro* in normal, starving and hypophysectomized animals [illegible]
of sulfate [illegible]
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inhibitory effect on sulfate (and acetate) incorporation in the chondrocytes and growth stimulation of the cells is a necessary condition for the appearance of the inhibiting actions of estrogens

Although the growth retarding effect of estrogens has been known for 30 years its mechanism and site of action still are not clear During the last decades a large variety of experiments have been carried out in order to explain the estrogen effects on body growth

The earliest hypothesis assumed that the estrogen-induced growth inhibition is due to a diminished production and/or release of growth hormone from the pituitary (Zondek 1936 Reece and Leonard 1939, Richards and Kueter 1941, Reiss *et al* 1946 Catt and Moffat 1967 Birge *et al* 1967 a) MacLeod *et al* (1969) demonstrated however that estradiol administration was without any effect on growth hormone synthesis in the pituitary

It has also been suggested that an increased secretion of ACTH after estrogen treatment might be responsible for the growth inhibition (Baker 1949)

Other investigators have suggested that estrogen-induced growth retardation is mainly due to a diminished appetite and restricted food intake of the animals

(Meites 1949, Aschkenasy-Lelu and Aschkenasy 1959) Metabolic alterations in the utilization of proteins and fat were also considered as contributory factors (Glasser 1954)

On the other hand, several workers suggested that estrogens exert their effects directly upon the growing tissues thus antagonizing peripheral effects of growth hormone (Schwartz *et al* 1969 a and b)

The interpretation of the estrogen growth hormone interactions became still more complicated when radio-immunoassay techniques detected increased plasma growth hormone levels after estrogen administration (Frantz and Rabkin 1965 a and b Merimee *et al* 1966, Carter *et al* 1970)

The present survey of estrogen effects on growth is a further attempt to elucidate the site and mechanism of action of the growth retarding and growth hormone antagonizing effects of estrogens

Materials and Methods

Animals, diet and operative procedures

Growing male or female mice of the NMRI strain were used throughout the study They were either purchased from Anticimex AB Sweden or they were selected from the colony of the institute which originated from animals obtained from Anticimex AB The mice were kept on wood shavings (obtained from different sources) in plastic cages and kept in a temperature controlled room (25° C) with 12 hrs of light and 12 hrs of darkness They had free access to commercial mouse pellets (Anticimex no 213) and tap water Vitamin supplement was given twice weekly (Protovit® Hoffman La Roche Basel Schweiz)

In the starvation experiments the mice were housed singly in metal cages with a wire mesh bottom only Coprophagy was not prevented and the animals had free access to water

Hypophysectomy was performed in 4 weeks-old mice (b w about 18 g) by a transauricular technique (Herbai 1970 a) The hypophysectomized mice were also supplied with a drinking solution containing 5 % glucose and 0 25 % NaCl The experiments were carried out 2 weeks after hypophysectomy and the sellar region was checked at autopsy under a dissection microscope (× 16) for completeness of hypophysectomy

Isotopes

Carrier free ³⁵S-labelled sodium sulfate and ³H labelled sodium acetate (sp a 500 mC/mmole) and ³H labelled phenol sp.a. 250 mC/mmole) were purchased from the Radiochemical Centre Amersham England Both tritiated compounds were dissolved in water and the stock solutions were stored in the frozen state until use

For determination of the calibration factor of the liquid scintillation spectrometer and for estimation of the counting efficiency standardized reference solutions of ³H hexadecane and lithium ³⁵S-sulfate were supplied from the Radiochemical Centre Amersham.

Chemicals hormones and drugs

Albumin human lyophilized purity grad more than 96 % of total protein content) was obtained from KABI AB Stockholm Sweden Unlabelled phenyl sulfate (A R grade) was purchased from Mann Res Lab U S A Papain EC 3 4 4 10 twice crystallized 2?3 mg of protein/ml of suspension was supplied from Sigma Chem Co St Louis Mo U S.A

17β-estradiol benzoate was donated by *Organon Co Oss Holland Diethylstilbestrol* (34 [illegible] No AKI 6321?1 P1 was purchased from ACO Stock[illegible] 10 triene 3 17β-diol) was obtained from Sigma Co [illegible] Ercostrol® Vallestril® 3-(6 methoxy 2 naphthyl) 2 2 [illegible] Ferro AB Stockholm Sweden The demethylation of methallenestril to its corresponding phenol was carried out by Dr I Terenius Department of Pharmacology Uppsala, Sweden who donated the compound for this investigation same batch as in Terenius 1963 In the *in vivo* experiments the estrogens were dissolved in and diluted with olive oil and injected subcutaneously

Porcine growth hormone (Somacton, batch No 6123; 1 IU/mg) was obtained from Ferring AB Malmo, Sweden It was dissolved in a mixture of 5 % glucose and 0.01 *M* phosphate buffer pH 7.4 (1 : 1; *v/v*). Ovine growth hormone (GH S9) was supplied by the Endocrino- [illegible] of carbonate- [illegible] 1, *v/v*). The [illegible]

In vivo experiments

[illegible] of the exchangeable inorganic sulfate pool of the mouse and the incorpora- [illegible] out by a double isotope method published [illegible] dure is briefly described here Four weeks- [illegible] 14 or 24 hrs after the last drug dose they were weighed and received an i.v. injection (0.1 ml) by the orbital plexus route (Pinkerton and Webber 1964) of the following injection mixture 20 μC ^{35}S with 96 μg (1 μmole) SO_4 and 20 μC ^{3}H-phenol with 376 μg (4 μmole) phenol dissolved in 0.1 ml 0.9 % NaCl During the preparation of the injection mixture samples were taken for determination of the ^{3}H and ^{35}S activities of the solution Thirty min after the injection of the mixture a urine sample (a few drops) was taken and the animals given 80 mg/ml $Na_2SO_4 \cdot 10 H_2O$ i.p. The urine sample was taken into a glass tube and evaporated to dryness in a vacuum-desiccator To the dry residue was then added 30 μl methanol containing 50 μg unlabelled phenyl sulfate/10 μl A 10 μl aliquot was transferred to the starting point of silica gel thin layer [illegible]

In vitro incubation experiments

After killing the animals with ether their chest cages were dissected free *in situ* The five [illegible]

of 25 μg/ml solution

After saturation of the incubation mixture with oxygen the flasks containing the cartilage samples and estrogen were shaken at 37° C for 2 hrs without labelled sulfate Thereafter 1 μC ^{35}S with 6 μg SO_4 per ml medium was added and the flasks shaken a further 2 hrs [illegible]

efficiency (DPM) This procedure permitted the expression of the results in picomoles of acetate and sulfate incorporated from the medium into the samples during the incubation period

TABLE I Effect of estradiol benzoate alone and combined with growth hormone (from two different cartilage of growing male mice Treatmant column shows daily dose of the compounds Significance test against controls * $p < 0\ 05$, ** $p < 0\ 01$, *** $p < 0\ 001$ The symbol growth hormone Controls received oil + vehicle

Exp	Treatment	n	Body weight at start g	Weight gain g	% of controls	Wet weight mg	
						ventral prostate	both testes
1	controls	5	15 3±0 1	+6 5±0 5	100	2 3±0 4	122±9
	20 µg EB	7	14 9±0 2	+3 4±0 6	52**	1 7±0 1	66±7***
	20 µg EB + 1 mg GH (porcine)	7	15 3±0 2	+2 7±0 5	41***	1 5±0 1*	90±8*§
	20 µg EB + 2 mg GH (porcine)	6	15 5±0 2	+3 0±0 4	46***	1 9±0 1	81±5**
2	controls	7	20 1±0 3	+3 7±0 3	100		
	200 µg GH (NIH ovine)	8	19 5±0 4	+3 4±0 2	92		
	100 µg EB	8	19 6±0 2	+3 0±0 3	81		
	100 µg EB + 200 µg GH (NIH ovine)	8	19 7±0 3	+2 3±0 3	62**		

Calculations

The sulfate pools and the incorporation rates of sulfate into the cartilage samples were calculated by a computer program Significance of differences between groups of data was tested by Student's t test In the cases of body weight changes sulfate pool values per g body weight and the DPM values per mg cartilage *in vitro* the statistics were calculated from data of the individual animals

Results

A *In vivo* studies

Effect of growth hormone in estrogen treated mice

It has been previously suggested that excessive doses of estrogens make rats functionally hypophysectomized and that growth hormone administration can be used as a replacement therapy for further growth (Reece and Leonard 1939 Richards and Kueter 1941 Griffiths and Young 1942) The present experiments were made to clarify whether oestrogen induced inhibition of body growth and depressed sulfate incorporation could be counteracted by simultaneous growth hormone treatment The results are summarized in Table I Twenty µg estradiol benzoate (EB) administrated for 6 days (exp 1) caused a considerable weight retardation and inhibition of sulfate incorporation while 100 µg EB for 3 days (exp 2) only diminished sulfate incorporation significantly Neither porcine nor ovine growth hormone (GH) in doses which had caused considerable stimulation of sulfate incorporation and body weight gain in hypophysectomized animals of the same strain (Herbai 1971 a), were able to increase the rate of sulfate incorporation or stimulate body weight gain of the EB treated animals As can be seen in the lowest line of Table I, the combination

species) on weight gain, size of sulfate pool and incorporation rate of sulfate *in vivo* into costal Duration of treatment 6 days in Exp 1 and 3 days in Exp 2 Figures are mean values and S E M § means significance test against the EB-treated group *EB = 17β-estradiol benzoate, GH =*

pituitary	Total sulfate pool µg	Sulfate pool per g b w µg	Incorporated sulfate per cartilage sample ng	% of controls
1 37±0 03	376±69	17 5±3 4	198±13	100
2 04±0 02***	395±29	21 6±1 6	98± 7	50***
1 77±0 02§§§	343±21	19 1±1 2	95± 9	48***
1 57±0 03§§§	309±28	16 7±1 4	110± 5	56***
	483±42	20 3±1 9	379±23	100
	427±22	18 6±0 8	290±16	76**
	409±23	18 1±1 0	216±12	57***
	395±40	18 0±1 7	182±11	48***

of EB and GH decreased weight gain more than did each of the hormones alone The size of the sulfate pool was more or less unaffected by the combination

EB elicited a decrease in the wet weight of the ventral prostate and testes (exp 1) and GH was unable to counteract this effect The weight of the pituitary was significantly increased after EB treatment, which is in agreement with previous reports (Noble 1938, Reece and Leonard 1939, Stevens and Helfenstein 1966) However, this increase was significantly reduced by GH This action indicates that the GH preparation was not inactive in these animals

Effect of stilbestrol on different kinds of cartilage

It has been previously shown that daily doses above 10 µg of stilbestrol like natural estrogens, considerably decreased the incorporation rate of sulfate into costal cartilage (Herbai 1970 e and 1971 b) The estrogen sensitivity of aural, xiphoid and tracheal cartilage was studied in order to ascertain whether the sulphate incorporation inhibiting actions of estrogens are confined to such cartilage that actively participates in skeletal growth The results shown in Table II indicate that all kinds of cartilage exhibited a dose dependent and rather similar decrease in sulfate incorporation rate after stilbestrol treatment

Effect of estrogen in starved animals

According to some previous reports growth retardation of animals after estrogen treatment is mainly caused by a decreased appetite and a subsequent food restriction (Meites 1949 Glasser 1954) In order to test the correctness of this hypothesis sulfate

TABLE II Effect of increasing doses of stilbestrol (6 days treatment) on the rate of sulfate incorporation *in vivo* into different kinds of cartilage in growing female mice The results are corrected for sulfate pool alterations and expressed as nanogram sulfate per trachea, xiphoid or costal cartilage sample and ng sulfate/mg ear cartilage Costal samples were handled individually and aural, xiphoid and tracheal samples were pooled for each group Figures in brackets show the values in % of cotrols Significance test against the control group ** = $p < 0.01$, *** = $p < 0.001$

Stilbestrol daily dose µg	n	Body weight before treatment g mean ± S E M	Weight gain g mean ± S E M	incorporation rate of sulfate into different kinds of cartilage			
				costal ± S E M	aural	xiphoid	tracheal
controls (oil)	6	14 8 ± 0 2	6 0 ± 0 3 (100)	342 ± 19 (100)	14 2 (100)	16 4 (100)	39 0 (100)
1	6	14 9 ± 0 1	6 0 ± 0 3 (100)	318 ± 7 (93)	9 5 (67)	11 1 (68)	30 8 (79)
10	6	14 7 ± 0 1	4 3 ± 0 4** (72)	168 ± 15***(49)	7 3 (51)	8 5 (52)	30 5 (78)
100	6	14 8 ± 0 1	1 5 ± 0 5*** (25)	134 ± 8***(39)	5 1 (36)	5 6 (34)	19 8 (51)
1000	7	14 7 ± 0 1	0 8 ± 0 3*** (13)	102 ± 7***(30)	4 3 (30)	3 5 (21)	16 1 (41)

incorporation was studied in starved mice which were treated in two separate experiments with two estrogens of different chemical structure The results are shown in Table III It can be seen in exp 1 that starvation caused a considerable weight loss of the animals but only a moderate decrease in the sulfate incorporation rate while a rather large dose of estrogen did not entirely prevent the weight gain but still depressed the sulfate incorporation rate below the starvation level Furthermore 17β-estradiol + 17β estradiol benzoate (given simultaneously to starving animals) elicited a highly significant decrease in the sulfate incorporation rate as compared

TABLE III Effect of starvation combined with estrogen treatment on weight gain size of sulfate E(f 17 β-estradiol (free alcohol), EB = 17β-estradiol benzoate "Treatment" values and S E M Significance test against controls * $p < 0.05$, *** $p < 0.01$ § tested

Exp	Duration of starvation	Treatment	n	Body weight at start g	Weight change g
1	none	controls	7	24 9 ± 0 6	+1 5 ± 0.2
	none	100 µg E(f) + 100 µg EB	8	26 5 ± 0 6	+1 2 ± 0 5
	38 hrs	controls	8	26 8 ± 0 4	−2 3 ± 0 1
	38 hrs	100 µg E(f) + 100 µg EB	7	25 6 ± 0 5	§−0 6 ± 0.2***
2	none	controls	4	18 0 ± 0 4	+2 6 ± 0 6
	48 hrs	controls	13	17 7 ± 0 2	−2 0 ± 0 2
	48 hrs	100 µg methallenestril	13	18 0 ± 0 2	−2 2 ± 0 2

to the starved non estrogenized animals The results of exp 2 show that methallenestril acted similarly to estradiol in starved animals A 2-day treatment caused a decrease (probably significant) in the size of sulfate pool of the mice, while *estradiol after a similar treatment* schedule had no such effect

B *In vitro* experiments

Effect of 17β estradiol and methallenestril phenol on cartilage from normal mice

Four week old female mice were killed and their lowest five pairs of attached ribs prepared as described above Diced pieces of the cartilaginous ribs including the osteochondral junctions were incubated for 2 hrs in the presence of 17β estradiol or methallenestril phenol in the incubation medium After adding ^{35}S sulfate to the solution the incubation was continued a further 2 hrs The results of the experiments are summarized in Table IV (exp 1, 2 and 3) As can be seen in Table IV, 17β estradiol and the free phenol of methallenestril markedly inhibited the ^{35}S sulfate uptake into costal cartilage both per whole sample and per mg cartilage In exp 1 all the available cartilage tissue of the standard rib sample was incubated, while in exp 2 only the osteochondral junctions with a small piece of adjacent 'resting' certilage was employed It is interesting that incubation of the "pure' osteochondral junction with *estrogen* resulted in a *considerably stronger* inhibition of sulfate uptake than that of the sample with large amounts of 'resting' cartilage This is due of the markedly higher uptake per mg in the 'pure' osteochondral junction In the presence of estrogen, the difference between the junction and 'resting' cartilage seems to have been eliminated It is also noteworthy that methallenestril phenol, which is about 10 times weaker as an estrogen than 17β estradiol (Terenius 1966 Herbai 1971 c), required only 2.5 times higher concentration in the medium for producing an equivalent inhibitory effect on sulfate uptake

pool and incorporation rate of sulfate *in vivo* into costal cartilage of growing female mice column shows daily dose which was given for 2 days Controls received oil only Figures are mean aga nst starved controls

Total sulfate pool µg	Sulfate pool per g b w µg	Incorporated sulfate per cartilage sample ng	% of non starved controls	% of starved controls
313±18	11 9±0 9	256± 6	100	—
287±22	10 3±0 7	152±12	60***	—
315±10	12 8±0 4	201± 7	79***	100
320±25	12 8±0 9	144± 6	56***	§71 6***
259±42	12 4±1 6	147± 5	100	—
228±17	14 4+1 0	62± 4	42***	100
§187±13*	§11 8±0 8*	44± 2	30***	§71***

TABLE IV Effect of 17β-estradiol and methallenestril phenol (—OH) in the incubation medium on female mice In exp 4 hypophysectomized mice were used Incubation time 2 hrs of 5 pairs of cartilaginous ribs with the osteochondral junctions (ribs No III—VII were incubated Figures are mean values and S E M Significance test against controls

Experiment	Estrogen in the incubation solution	n	Body weight of the mice g	DPM^{35}S in the whole cartilage sample	% of controls
1	no estrogen	5	17 9±0 3	221,200±33 600	100
	17β-estradiol 10 μg/ml	5	17 9±0 4	147,400±11,400	66 6*
2	no estrogen	5	17 7±0 4	98,400±14,000	100
	17β-estradiol 10 μg/ml	5	17 6±0 4	39,800± 4,900	40 4**
3	no estrogen	4	17 3±0 3	347,000±13,600	100
	methallenestril OH 25 μg/ml	4	17 3±0 4	137 000±10,900	39 5***
4	no estrogen, normal mice	5	29 1±0 6	158,200±14,500	100
	no estrogen, hypophysectomized mice	5	22 0±0 5	88,700±5300	56 1***
	methallenestril OH 25 μg/ml, hyp mice	5	21 0±0 7	83,500±6800	52 8***

Effect of methallenestril-phenol on cartilage from hypophysectomized mice

As expected costal cartilage pieces from hypophysectomized mice showed markedly less sulfate incorporation *in vitro* than controls (Table IV exp 4) The decrease in the present experiments was similar to that previously obtained *in vivo* (Herbai 1971 a) However if costal cartilage from hypophysectomized mice was incubated with methallenestril phenol, no further depression of sulfate incorporation could be achieved by the estrogen (exp 4 of Table IV) The results were similar whether the whole cartilage sample was considered or the results expressed on a dry-weight basis

Effect of methallenestril phenol on incorporation rate of ^{35}S-sulfate and ^{3}H acetate into cartilage pieces

Since sulfation and acetylation of the newly synthetized chondroitin sulfate chain are two different biochemical reactions it was of interest to investigate whether estrogens only inhibit the sulfation or also affect acetylation of the polysaccharide The results of a double isotope experiment with ^{3}H acetate and ^{35}S sulfate in the presence of methallenestril phenol in the incubation solution are given in Table V It is clearly seen that both sulfation and acetylation were parallelly affected by the presence of estrogen This is true both with regards to the whole cartilage sample of a flask or on a dry-weight basis *Both reactions were depressed to the same extent* and the ^{3}H/^{35}S ratios were indentical in the estrogen-containing and the control solutions Since the specific activities of the labelled materials in the incubation medium were known and the absolute counting efficiencies of the tracers could be calculated the

on the incorporation rate *in vitro* of ^{35}S sulfate into diced costal cartilage pieces of growing with estrogen without labelled sulfate and then 2 hrs with ^{35}S sulfate Each flask contained pieces from one mouse In exp 2 only the osteochondral junctions with a small piece of adjacent cartilage
$^{*}p < 0\ 05$, $^{**}p < 0\ 01$, $^{***}p < 0\ 001$

Dry weight of the sample mg	DPM ^{35}S per mg cartilage	% of controls
7 5 ±0 8	29 100±1300	100
6 4 ±0 4	23 000±1600	79**
1 82 ±0 14	53 200±4900	100
1 98 ±0 15	19 800±1150	37 2***
11 8 ±0 4	29,800±1900	100
12 3 ±0 4	11,200± 900	37 6***
7 5 ±0 4	20 900±1400	100
7 1 ±0 2	12 500± 690	59 8***
6 7 ±0 3	12 400± 540	59 3***

TABLE [illegible]

in picomoles

	n	Body weight g	DPM per total cartilage sample ^{35}S	DPM per total cartilage sample ^{3}H	Dry weight of cartilage sample mg	DPM per mg dry cartilage ^{35}S	DPM per mg dry cartilage ^{3}H	^{3}H/^{35}S
controls no estrogen	5	20 3±0 5	52 800 ±7 400	140 000 ±9 300	9 1±0 4	5760 ±620	15 450 ±920	2 8
			(1,500)	(127)		(164)	(14)	
25 μg methallenestril phenol/ml incubation medium	5	21 0±0 5	25 800** ±580	72 200*** ±6 600	8 9±0 9	2970** ±210	8460** ±1 250	2 8
			(753)	(66)		(84)	(8)	
per cent of control value			49	52		52	55	

results were also expressed in chemical terms, as picomoles sulfate and acetate incorporated during the incubation period It can be seen in Table V that approximately 10 times more exogenous sulfate than acetate was incorporated from the medium into the cartilage samples These relations are in good agreement with previous sulfate acetate experiments *in vitro* of Boström and Månsson (1952) and Herbai and Lindahl (1970)

Discussion

The well established estrogen induced retardation of body growth of laboratory animals has been ascribed to an anti growth hormone effect of the estrogens (Zondek 1936 Gaarenstroom and Levie 1939 Richards and Kueter 1941, Griffiths and Young 1942 Reiss *et al* 1946) A tempting hypothesis was to consider the animals which had been growth retarded by estrogens, as functionally hypophysectomized with a possible elimination by growth hormone substitution of the estrogen induced changes However, administration of growth promoting pituitary extracts did not yield uniform results Gaarenstroom and Levie (1939) obtained stimulation of weight gain and growth of tail length by pituitary extract in estrone treated, but not in stilbestrol treated rats In contrast Richards and Kueter (1941) reported a successful weight gain stimulation by a pituitary extract in stilbestrol treated rats Furthermore Griffiths and Young (1942) and Reiss *et al* (1946) obtained a dissociation between the weight gain and tail stimulating effects of their growth hormone preparations in estrogen treated animals Their growth hormone preparations stimulated body weight gain but failed to affect the arrested tail growth

In the present experiments much more purified porcine and ovine growth hormone preparations were used Both of them in a previous study markedly stimulated body weight gain and sulfate incorporation rate in hypophysectomized mice of the same strain as used in the present study (Herbai 1971 a) As seen in Table I neither porcine nor ovine growth hormone counteracted the estradiol benzoate induced body weight retardation or inhibition of sulfate incorporation This lack of effect of growth hormone makes it evident that the estrogen induced changes cannot depend on a deficiency in the hormone The apparent stimulating effects of the hypophyseal growth promoting preparations in earlier work might have depended on pituitary principles other than growth hormone since the preparations available at that time were probably less pure than the present ones

According to the present results it is improbable that estrogens act on sulfation through a decreased growth hormone synthesis or release in the pituitary This is supported by recent reports that estradiol administration to rats failed to decrease growth hormone synthesis in the pituitary (Birge *et al* 1967 b MacLeod *et al* 1969)

It is surprising that 200 μg ovine growth hormone significantly depressed sulfate incorporation compared to normal controls Table I exp 2) One possible explanation is that the injected growth hormone obtained from species other than mice is able to block the production of the endogenous growth hormone of the

mouse by a negative feed back mechanism (Müller and Pecile 1966, Sakuma and Knobil 1970), but is too weak in the mouse to produce observable effects Another explanation could be competition between the exogenous less efficient hormone and the own growth hormone for GH-sensitive receptor sites in the cartilage cell

The starvation experiments of Table III clearly indicate that both 17β-estradiol and methallenestril in addition to the starvation dependent decrease of sulfation, further inhibited sulfate incorporation The effect thus being independent of appetite and food intake of the animals The smaller weight loss after estrogen than after oil (exp 1) in the starved mice, which seems paradoxal, might be due to a sodium and water retention, such an effect has repeatedly been observed after estrogen administration (Fisher 1954, Woolley and Timiras 1964)

It has been reported that estrogens still cause a variety of beneficial metabolic actions without affecting plasma growth hormone levels in acromegalic (Schwartz *et al* 1969 a) and osteoporotic patients (Schwartz *et al* 1969 b) These metabolic actions could be compatible with a peripheral antagonizing action of estrogens upon the growth hormone effects In the present *in vitro* experiments (Table IV and V), very convincing evidence was obtained for a peripheral action of the estrogens The results suggest that estrogens block some growth hormone mediated anabolic reactions in the growing cartilage cell As far as we know, similar experiments have not been previously published It can be mentioned that the concentration of 17β estradiol in the incubation medium which contained 2 % albumin, is of a similar order of magnitude as would be expected from those estradiol benzoate doses which elicited a similar degree of sulfate incorporation inhibition *in vivo* (*cf* Herbai 1970 e and 1971 b)

Autoradiographic studies in costal cartilage of growing mice revealed marked accumulation of ^{35}S *in vivo* at the osteochondral junction, as compared to the remaining 'resting' part of the rib (Herbai 1970 d) Estrogen treatment caused a marked narrowing of this active junction region and a reduced ^{35}S uptake The present *in vitro* results (Table IV, exp 1 and 2) confirm quantitatively both the increased sulfation activity in the osteochondral junction and the marked sulfation inhibiting effect of the estrogens As seen in Table IV the osteochondral junctions incorporated much more ^{35}S sulfate per mg sample than did the mixed 'diluted' cartilage pieces and it is interesting that estradiol decreased the rate of incorporation in both groups to a similar level If the values derived from the osteochondral junctions are subtracted from the data of the mixed cartilage, it becomes obvious that estradiol has a negligible inhibitory effect on the 'resting' part of the cartilage This finding suggests that estrogens, as well as the lack of the growth hormone following hypophysectomy, reduce sulfate incorporation most intensely in the osteochondral region, which is known to be the main site for longitudinal growth of bone

Apparently contrasting data have been obtained in Table II, where stilbestrol decreased sulfate incorporation not only in costal but also in aural, xiphoid and tracheal cartilage which all lack cartilage bone junctions and never undergo ossifica

tion This strong stilbestrol effect upon non ossifying cartilage might be explained by findings which suggest that stilbestrol action on growing cartilage is qualitatively different from that of other estrogens (for detailed evidence see Gaarenstroom and Levie 1939) Furthermore, it was observed in the same study and by Richards and Kueter (1941) that when stilbestrol was supplied in doses equivalent to estradiol it caused marked liver and kidney damage and hemorrhages in the adrenal cortex These observations suggest that the cause of the general depression of sulfate incorporation in all types of cartilage (Table II) may be different from the inhibitory effect elicited by other estrogens in growth hormone stimulated tissues An important argument for the validity of this hypothesis is the fact that hypophysectomy (Herbai 1971 a) depressed sulfate incorporation in all the kinds of cartilage employed in the experiment shown in Table II, but growth hormone was only able to stimulate the sulfation reaction in costal cartilage which contains osteochondral junctions

In relation to its estrogenicity methallenestril phenol was a stronger inhibitor of sulfate incorporation *in vivo* than estradiol benzoate (Herbai 1971 c) and the validity of this finding has been extended in the present study *in vitro* (Table IV exp 3)

In cartilage from hypophysectomized mice the sulfate uptake was much lower than in normal controls This agrees with previous *in vivo* findings (Herbai 1971 a) Methallenestril phenol was unable to further depress sulfate incorporation in cartilage pieces from hypophysectomized mice This indicates that growth stimulation of cartilage is a condition for inhibitory effects of estrogens Whether this growth stimulation has to be directly due to growth hormone (perhaps mediated through the sulfation factor) or may be induced by some other factor is not clear Paesi and de Jongh (1954) in very young hypophysectomized rats (29—35 g) found an inhibition of body weight gain and tail length increase by estradiol benzoate According to Noble (1938) such young rats (under 100 g) do not need growth hormone for growth In the present study the hypophysectomized mice were allowed to reach a weight plateau well before the *in vitro* experiment Thus they lacked growth hormone and consequently estrogen had no inhibitory effect To decide the question whether the effect of estrogen is directly growth hormone dependent cartilage pieces from hypophysectomized but still growing animals (Paesi and de Jongh 1954) should be tested for estrogen sensitivity under our *in vitro* conditions

The similar degree of decrease in the incorporation rates of sulfate and acetate in the presence of estrogen shows that not only the sulfation process but also another step involved in the synthesis of chondroitin sulfate is estrogen sensitive The high sulfate/acetate ratio in the cartilage samples both with and without estrogen suggests that sulfate is incorporated mainly from the incubation medium while acetate to a large extent is supplied from endogenous sources

The results obtained in this study unequivocally indicate that estrogen induced inhibition of growth is not due to growth hormone deficiency or to the anorexigenic effect of the hormone Furthermore it can be concluded that estrogens have a local action probably on the chondrocytes While it seems probable that a growth hormone

stimulated process is a necessary condition for estrogenic action on the synthesis of chondroitin sulfate further biochemical work is necessary for the clarification of the exact site and mechanism of estrogenic action upon the osteochondral junction

I am grateful to Dr L Terenius for valuable advice and donation of the demethylated methallenestril preparation and to Professor E Barány for his stimulating interest and criticism The skilful technical assistance of Mrs B Jansson is gratefully appreciated Thanks are due to Prof Wilhelmi Emory University Atlanta Georgia for generous supply of ovine GH

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The Influence of Noradrenaline Synthesis Inhibition on the Disposition of ³H-Metaraminol in Peripheral Adrenergic Nerves

By

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Abstract

ALMGREN, O *The influence of noradrenaline synthesis inhibition on the disposition of ³H-metaraminol in peripheral adrenergic nerves* Acta physiol scand 1971 *83* 91—95

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occurs

Injected metaraminol (MA) accumulates in the adrenergic nerve terminals of sympathetically innervated organs and thereby displaces stoechiometric amounts of noradrenaline (NA) (Andén 1964, Shore, Busfield and Alpers 1964) The function of the peripheral adrenergic nerves seems not to be affected, even when as much as 95 per cent of the endogenous transmitter pool is thus displaced by a much weaker agonist (Anden *et al* 1964) This could indicate that the release of NA by nerve impulses is sufficient also when the pool is very small Such a view is also supported by the analysis of the urinary excretion of NA at different time intervals after MA administration performed by Anden *et al* (1965) In order to further elucidate the handling of MA by the nerve terminals, a study of the disposition of small amounts labelled MA was performed (Almgren and Waldeck 1967) It was found that ³H-MA disappeared in 2 exponential phases, one initial rapid phase and one second, slower phase When the nerve impulse flow was broken by decentralization the disappearance of the amine followed a single exponential course, much slower than the

TABLE I Correlation and regression coefficients for the values presented in Fig 1 All regres. coefficients are significant at $p<0.01$

		Intact gland	Decentr gland	Heart
Control	Time period (hrs)	1—36	1—48	1—48
	N	41	44	41
	Correl. coeff	—0 97	—0 84	—0 76
	Regr coeff	—0 0267	—0 0093	—0 008?
H 44 68	Time period (hrs)	1—48	1—48	1—48
	N	38	38	33
	Correl coeff	—0 77	—0 50	—0 61
	Regr coeff	—0 0118	—0 0045	—0 0056

initial, rapid phase of the intact gland As the rate of NA synthesis is dependent on the nerve impulse flow (Roth Stjarne and Euler 1966, Sedvall and Kopin 1967 Sedvall Weise and Kopin 1968) it was considered of interest to investigate if inhibition of the NA synthesis would affect the disappearance of MA

Methods

Male Sprague Dawley rats weighing about 150 g were unilaterally decentralized (Almgren and Waldeck 1967) 1 week before the experiment To prevent hypothermia the rats were kept at a constant temperature of 29° C throughout the whole experiment 40 μg/kg of dl-metaraminol 7 ^{3}H (specific activity 1 63 C/mM New England Nuclear Chemicals) was injected in a tail vein To the experimental group 500 mg/kg of DL-α methyl tyrosine methyl ester (H 44/68) was given i p 1 h before the amine injection Repeated doses of 250 mg/kg of this drug were given each 12 h After about 24 h the rats had a marked diarrhoea and refused eating and drinking To compensate for loss of body fluid repeated i p injections of isotonic NaCl were given The control rats received at the time of H 44/68 administration in the experimental group isotonic NaCl i p At certain intervals after the administration of ^{3}H MA (see Fig 1 the rats were killed by a blow on the head and the hearts and submaxillary plus sublingual glands of both sides were taken out, weighed and homogenized in ice-cold 0 4 N perchloric acid After centrifugation and filtration the extracts were mixed with equal amounts of Insta-Gel Emulsifier (Packard Instrument Company Inc) and total radioactivity was measured in a Packard Tricarb Liquid Scintillation Counter Correlation and linear regression was calculated according to Davies p 118 f f (1949) The coefficients are presented in Table I The lines in the figures were drawn on the basis of the linear equations calculated The significance of the difference between slopes was calculated with an analysis of variance In a few instances Student s t test was used to estimate differences at certain time intervals

Results

In Fig 1 the ^{3}H MA content of hearts and intact and decentralized salivary glands at different time intervals after an i v injection of 40 μg/kg is presented in a semi logarithmic graph There was no significant difference in uptake of ^{3}H MA between intact and decentralized glands The disappearance of the amine was faster in the intact than in the decentralized gland during the first 36 h ($P<0.001$) The decline of the ^{3}H MA level during this period seemed to be exponential with a half life of about 11 h After that time the disappearance was retarded (half life about 50 h) In the decentralized gland on the other hand there seemed to be a single exponential decline of the ^{3}H MA level The half-life was about 32 h Also in the heart the decline of the ^{3}H-MA level seemed to be exponential with a half life of about 37 h.

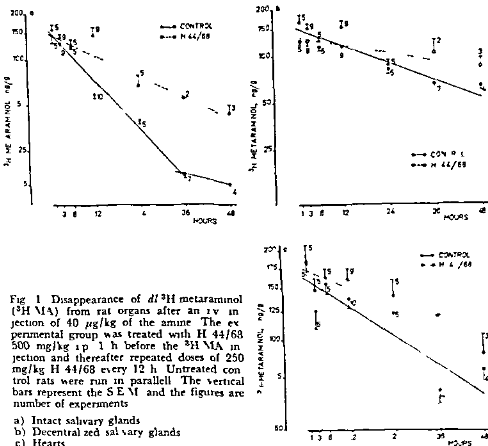


Fig 1 Disappearance of *dl* ^{3}H metaraminol (^{3}H MA) from rat organs after an i v injection of 40 μg/kg of the amine The experimental group was treated with H 44/68 500 mg/kg i p 1 h before the ^{3}H MA injection and thereafter repeated doses of 250 mg/kg H 44/68 every 12 h Untreated control rats were run in parallell The vertical bars represent the S E M and the figures are number of experiments

a) Intact salivary glands
b) Decentralized salivary glands
c) Hearts

It did not significantly differ from the decline of ^{3}H MA found in the decentralized gland

When 40 μg/kg ^{3}H MA was given i v 1 h after the i p administration of 500 mg/kg H 44/68 there tended to be a small although not significant reduction of uptake most marked in the decentralized gland 6 h after the administration of H 44/68 the rats appeared essentially normal There was only a slight decrease in spontaneous motility and a moderate diarrhoea although no weight reduction Also at this time interval there were no significant differences between the ^{3}H MA contents of glands and hearts in H 44/68 — treated and untreated animals 12 h after the injection of H 44/68 there was still no weight reduction of the animals The gross appearance was the same as after 6 h At his time interval however significantly higher amounts of ^{3}H MA were found in the salivary glands of the H 44/68-treated animals than in the controls ($P<0.001$ in intact glands and $P<0.025$ in decentralized glands) The same tendency was noted in the hearts but was found not to be significant

When the inhibition of NA synthesis was maintained by repeated injections of H 44/68 the ^{3}H MA disappearance from the intact glands seemed to follow a single

exponential course during the time period studied (Fig 1 a) It was also markedly retarded ($P<0\ 001$), with a half-life of about 26 h However, it was still faster than the ^{3}H-MA disappearance from the decentralized gland ($P<0\ 001$) or from the heart ($P<0\ 005$)

The disappearance of ^{3}H-MA from the decentralized gland (Fig 1 b) still after H 44/68 treatment seemed to follow an exponential course, but was retarded as compared to decentralized glands from untreated rats ($P<0\ 005$), with a half life of about 67 h Also the decline of the ^{3}H MA level in the heart (Fig 1 c) seemed to follow an exponential course it was slightly though not significantly retarded by H 44/68 treatment

Discussion

The disappearance of MA from sympathetically innervated tissues is slow compared to noradrenaline (*cf* Fischer and Snyder 1965, Almgren and Waldeck 1967, Crout *et al* 1964, Iversen 1967) The reason for this is generally ascribed to the resistance of MA to the catabolizing enzymes monoamine oxidase and catechol O methyl transferase

The disappearance of ^{3}H-MA from the salivary glands is dependent on the physiological nerve impulse flow (Almgren and Waldeck 1967 Fig 1, this paper) It has been proposed that accumulated MA is redistributed from a "readily releasable" pool to a pool where the nerve impulses are less effective in releasing the amine (Crout *et al* 1964) This could explain the two phases of disappearance from intact salivary glands found earlier (Almgren and Waldeck 1967) and indicated also in the present study

The very slow disappearance of ^{3}H-MA from the heart found in the present study is in accordance with the findings of Crout *et al* (1964) This is probably due to a low impulse flow in the adrenergic nerves of this organ under the present conditions Support for this view is given by Corrodi and Malmfors (1966) who found no decrease in endogenous NA levels in rat hearts 12 h after the administration of a synthesis inhibitor if the rats were kept in room temperature (22° C) When kept in a cold environment (4° C) a decrease was observed Also the rate of NA synthesis is normally low about half of that found in submaxillary glands (Sedvall *et al* 1968) In the present study with rats kept at 29 C it was not possible to show any significant decrease in *^{3}H MA disappearance after H 44/68 treatment*

From both intact and decentralized salivary glands, however the disappearance of ^{3}H MA was markedly reduced when the synthesis of NA was inhibited During the time period studied 48 h the disappearance of ^{3}H-MA seemed to follow single exponential courses in both glands

Several possible explanations for the retarded disappearance of ^{3}H MA exist The condition of the H 44/68-treated animals might be a factor of importance The fact, however, that the retardation by H 44/68 treatment of the ^{3}H MA disappearance from the salivary glands was obvious already after 12 h when the gross appearance of the animals was still normal, strongly contradicts this explanation A central in

hibition of the impulse flow induced by the drug itself might also be considered However, since there was a significant retardation of the ^{3}H-MA disappearance also from the decentralized glands this is not very likely Besides, endogenous NA disappears more rapidly from intact than from decentralized rat submaxillary glands after administration of a synthesis inhibitor (Corrodi and Malmfors 1966) indicating the presence of an impulse flow in adrenergic nerves of intact glands after synthesis inhibition

Another possibility to consider is that a major part of ^{3}H-MA disappearance normally is caused by displacement from storage sites by newly synthesized NA Injected ^{3}H MA appears to be initially localized in the same granular sites as newly synthesized NA, but is gradually transferred to more stable storage sites (vide supra) When the catecholamine synthesis is inhibited the amount of NA available for displacement of ^{3}H MA is reduced and thus the loss of the latter amine is retarded

The retardation of ^{3}H MA disappearance was most marked in the intact glands during the first, normally rapid, phase It was, however, also apparent in the de- [illegible]

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Renal Vascular Resistance in Spontaneously Hypertensive Rats

By

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Abstract

FOLKOW B M HALLBÄCK Y LUNDGREN and L WEISS *Renal vascular resistance in spontaneously hypertensive rats* Acta physiol scand 1971 83 96—105

The renal vascular beds of one spontaneously hypertensive rat (SHR) and one matched normotensive control rat (NCR) were perfused with oxygenated plasma substitute in 27 paired expts Pressure flow curves indicated that the renal vascular resistance is lower in SHR than in NCR during maximal dilatation in contrast to the situation in other major circuits while the SHR renal resistance vessels are less distensible than those of NCR —Graded noradrenaline (NA) infusions showed that the NA threshold does not differ significantly between the two groups but SHR displays a steeper dose response curve and an increased maximal pressor response as compared to NCR —The results suggest an increased media thickness in the SHR renal resistance vessels raising their wall/lumen ratio reactivity and maximal contractile strength but reducing their distensibility However while the thickened vascular walls in other major circuits encroach upon the lumina even at maximal dilatation (Folkow *et al* 1970 a b) they are in the kidneys combined with increased vascular lumina This implies a larger renal blood supply in SHR than in NCR at very low level of vascular tone while the reverse is true at enhanced tone due to the steeper resistance curve in the SHR

Renal resistance in essential hypertension of man has repeatedly been studied by a large number of investigators and it has been established to be increased also during rest (for ref see Pickering 1968) It is however so far not known whether this increase is due to a structurally determined vascular narrowing to extrinsic excitatory influences on the renal vessels or if it simply is a consequence of the pronounced autoregulatory capacity of these vessels

In the present study spontaneously hypertensive rats (SHR) which seem to provide the best animal model so far of human essential hypertension have been used to explore renal hemodynamics Earlier studies of this animal model with respect to the resistance of the entire systemic vascular bed and also that of the hindquarters (Folkow *et al* 1970 a b) have shown that it is increased even at maximal dilatation in SHR almost in proportion to their raised resting arterial blood pressure Further at constant flow perfusion the resistance responses of the hindquarters to graded noradrenaline (NA) doses showed a steeper dose response curve and an increased maximal pressor responce in SHR as compared with NCR but no

difference in NA threshold All these findings can be explained by a structural change of the SHR resistance vessels, with an increased media thickness encroaching upon the vascular lumina even at maximal dilatation This structural change appears to be so pronounced that it may alone cause the raised arterial pressure during rest as well as the raised vascular reactivity" in SHR Further, earlier studies on regional vascular beds in man (forearm and hand, Folkow 1956, Folkow, Grimby and Thulesius 1958, Conway 1963, Sivertsson 1970) strongly suggest that a similar type of change accounts for largely the entire increase of systemic flow resistance in resting subjects with essential hypertension. In addition most morphological studies of small arteries and arterioles in hypertensive subjects indicate the presence of media hypertrophy, particularly well documented in a fairly recent study by Furuyama (1962)

Since the renal vascular bed has attracted particular interest in essential hyper tension as well as in other types of hypertension in man, it has been subjected to a detailed hemodynamic analysis in the present study on SHR. In order to minimize, in this first study, the influence of such vasoexcitatory stimuli bloodborne or neurogenic that are present in the intact animal, the isolated renal vascular bed was perfused with a plasma substitute, starting from the level of maximal vasodilatation The resistance responses to graded NA doses were then compared in SHR and matched NCR up to the level of maximal constriction using the same constant perfusion technique as in earlier studies of the hindquarter vessels (Folkow *et al* 1970 b) to explore whether the renal resistance vessels display similar structural changes as those met with in the hindquarter vessels The results have been presented earlier in a preliminary report (Folkow *et al* 1971)

Methods

27 paired perfusion expts were performed on spontaneously hypertensive rats (SHR) of the Wistar strain (Okamoto 1963) and matched normotensive control rats (NCR) about 7 months of age and of both sexes The 2 animals were anesthetized by means of an i p injection of Nembutal 3—4 mg/100 g b w and tracheal cannulas were inserted to permit free airways After heparin zation arterial pressure was measured from one of the femoral arteries *via* polyethylene tubes utilizing Statham pressure transducers recording on a Grass polygraph Model 7

In identical and largely simultaneous preparations the 2 animals were eviscerated and the abdominal aorta was freed for 10 mm distally and 5 mm proximally to the renal arteries Great care was taken to ligate *all* branches along this part of the aorta except the two renal arteries A perfusion cannula was then inserted in the distal part of the aorta towards the renal arteries and a ligature was loosely placed around the aorta just proximally to these arteries Thus two preparations each consisting only of the renal vascular beds in the two animals respectively were achieved When the perfusion of the kidneys was started the aortic ligatures proximally to the kidneys were tightened the animals were killed and the inferior caval veins were widely opened at the level of the renal veins to permit free outflow

The perfusate consisted of oxygenated Tyrode solution containing 3 % of an artifical colloid (Ficoll a synthetic polymer of sucrose and epichlorohydrin, kindly supplied by AB Pharmacia Sweden) to maintain a reasonably normal colloid osmotic pressure thus minimizing edema formation The perfusate emerged from a common container kept at 38° C and drained by a single tube coupled in series with a small mixing chamber that was steadily shaken The efferent tube from this mixing chamber branched into two tubes each of which after passing through a double Harward perfusion pump was connected to the respective aortic cannula The turbulence created in the mixing chamber had the advantage that vasoactive

agents when administered proximally to the chamber could be injected or infused in identical concentrations to the two renal preparations. The perfusion pump was adjusted so as to deliver 60—70 ml of perfusate per 100 g of renal tissue each minute and it was repeatedly checked that the perfusion rates of the two preparations were *identical and constant*. The arterial inflow pressure was measured *via* side tubes placed just proximally to the two aortic cannulas and connected to two Statham pressure transducers writing on a Grass recorder Model 7. The transducers were placed at the aortic level; venous outflow pressure at the cut caval veins was considered to be zero. When after a 10—15 min period of perfusion the aortic pressure had stabilized 0.25—1.0 mg of papaverine was added to the perfusate as slug injections to secure complete relaxation of the renal vascular smooth muscles.

From measurements on the maximally dilated renal vascular beds pressure flow curves were constructed for 11 SHR and 11 matched NCR where the renal capsules had been left intact, and for 4 pairs of decapsulated SHR and NCR kidneys. For this purpose the rate of the perfusion pump was changed in a random fashion from a "basal" flow of 60—70 ml up to about 500 ml/min/100 g tissue while the resulting pressure changes were recorded.

In another series of experiments on 12 pairs of SHR-NCR kidneys graded doses of noradrenaline (NA) dissolved in the perfusion medium were administered as a constant infusion starting from the level of maximal dilatation. From barely subthreshold NA dosages the *NA concentration was then increased in a stepwise fashion as soon as a steady state response* for the previous dosage had been reached until the maximal obtainable pressor response had been achieved. This was checked by adding slug injections of huge NA doses (0.5—1 mg) and in addition supramaximal doses of vasopressin (10 I.U.) and slug injections of huge doses (50—100 mg) of $BaCl_2$ were added. The local concentrations of the vasoactive agents could be considered to be equal in the two preparations since equal flows per unit tissue mass were used.

After the experiments India Ink was sometimes injected intraarterially and the kidneys were always carefully inspected to check that all parts had been perfused, after which they were weighed. Kidneys of other SHR and NCR of equal body weights and age, but not exposed to artificial perfusion, had been weighed in an earlier series to give normal average figures for the relationship between renal mass and body weight. Comparisons between these figures and those from the artificially perfused kidneys showed that edema formation was usually very slight as a result of the artificial perfusion.

Results

Resting arterial blood pressure as measured in the femoral artery of the SHR and the NCR differed significantly ($p < 0.001$) being 175 ± 10 and 119 ± 5 (SEM) respectively, in agreement with previous results (Folkow *et al.* 1970 a).

The compiled results of pressure flow curves constructed from the maximally dilated vascular beds of 11 pairs of simultaneously perfused SHR and NCR kidneys are shown in the left part of Fig. 1. Note that the curve representing the SHR kidneys indicates a significantly lower renal vascular resistance at maximal dilatation as compared to that of NCR, particularly at lower pressures. At higher perfusion (and *transmural) pressures, on the other hand, the two pressure flow curves approach each other since the NCR resistance vessels evidently are more distensible than the* SHR ones (see also below).

In the right part of Fig. 1 the pressure flow values from the left part are recalculated to relate pressure to resistance during maximal dilatation. These latter curves illustrate not only the lower renal vascular resistance in SHR but also and more directly than the left hand curves the reduced distensibility of the SHR resistance vessels as compared with those of the NCR. Thus the resistance ratio between the pressures of 40 and 60 mm Hg was 1.0 in the SHR renal vascular bed and 1.16 in that of the NCR.—The decapsulated kidneys showed largely the same pressure flow patterns which indicates that the lower renal resistance in SHR a

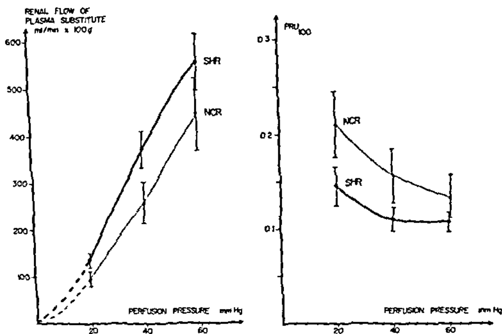


Fig 1 The left part presents the mean pressure flow curves in the maximally dilated renal vascular beds of 11 pairs of simultaneously perfused SHR and NCR The right part shows the same values, recalculated to related pressure to resistance Vertical bars indicate SEM The curves are significantly separated ($p < 0.001$)

maximal dilatation could hardly be explained by any difference in intracapsular pressure

In 12 simultaneously perfused SHR and NCR kidneys noradrenaline (NA) was infused from subthreshold to supramaximal doses during constant flow perfusion starting from the level of maximal vascular relaxation The resistance (pressor) responses were related to the log NA dose to give the dose-response curves For each paired experiment the difference between the SHR and NCR curves was calculated and the compiled results of all the experiments are summarized in Fig 2 During complete relaxation of the renal vascular smooth muscles, the mean resistance of the renal vascular bed was also here lower in SHR than in NCR being 0 24 PRU_{100} and 0 32 PRU_{100}, respectively, at a flow level of about 70/min/100 g Like the pressure-flow curves this implies the presence of a structurally determined difference in vascular lumina between SHR and NCR and its extent corresponds well with that shown in the left part of Fig 1

There was no difference between SHR and NCR with respect to threshold sensitivity to NA, defined as the NA dose producing a 25 per cent increase of resistance above that at maximal dilatation However, once more substantial vasconstrictions were initiated by higher NA doses, the SHR vessels responded in an accelerated manner compared to the NCR vessels, resulting in a crossing over of the two

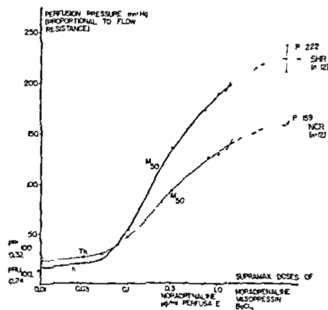


Fig 2 Constant flow perfusion of the parallel coupled renal vascular beds of 12 SHR and 12 NCR showing mean values of resistance responses to graded noradrenaline doses Th denotes threshold, i e a 25 per cent increase of resistance above the state of complete vascular relaxation M_{50} denotes 50 per cent of the maximal response (compare Folkow *et al* 1970 b)

resistance curves (Fig 2) This implies an increased steepness of the SHR resistance curve the corrected tangent of the angle being about 135 % larger in SHR than NCR ($p < 0.005$) In addition supramaximal NA doses produced an about 40 per cent larger pressor resonse in the SHR renal vascular bed than in that of the NCR ($p < 0.001$) Definite maximal responses could however, be achieved first when supramaximal doses of vasopressin or Ba, were added as well Thus also to these constrictor agents the SHR vessels displayed a 40 % larger pressor response ($p < 0.001$) indicating a clearly higher maximal contractile strength of the SHR renal resistance vessels

Discussion

Since Goldblatt's classical studies the kidneys have attracted great interest with respect to their possible etiological role in hypertensive disease in man (for ref see Pickering 1968) Earlier assumptions that a primary interference with renal blood supply should be quite a common causative element in hypertensive disease in general have not been corroborated though such interferences are no doubt present in a distinct but limited group of cases However in the most common type of this disease essential (primary hypertension there is no clear evidence of any truly abnormal interference with renal blood supply in the early uncomplicated stage This by no means denies that such interferences may be common once secondary vascular lesions develop Such a distinction between primary and secondary renal vascular changes is of course most important when the etiology of essential hypertension is discussed

Clearance studies in man indicate a raised renal flow resistance during hypertension However this is to be expected for purley physiological reasons because of the very efficient blood flow autoregulation in the kidneys and cannot alone be taken as evidence of any pathological interference with renal blood supply In case a maximal renal vasodilatation easily could be induced in man information could be gained about possible structurally based differences in renal resistance, in analogy with findings in the forearm and hand of subjects with essential hypertension (for ref see *e g* Sivertsson 1970) It is further difficult to analyze in detail the highly complex intrarenal vascular events with the aid of clerance measurements alone, which are the only methods available in man

In order to bypass such methodological difficulties inherent in human experimental research the genetically hypertensive rat might provide an animal model relevant also for early stages of human essential hypertension In the present experiments renal flow resistance was compared in spontaneously hypertensive rats (SHR) of relatively low age (6—7 months) and in matched normotensive control rats (NCR), from maximal dilatation to maximal vasoconstriction In comparison with NCR the SHR group displayed the following characteristics of the renal resistance vessels

1) About 30 per cent lower flow resistance during maximal dilatation when low perfusion pressures were used ($p < 0.001$ when both the pressure flow studies and the dose response studies were considered)

2) Reduced distensibility of the SHR resistance vessels implying that the above mentioned difference grew smaller at higher levels of transmural pressures

3) Unchanged sensitivity to threshold amounts of vasoconstrictor agents

4) Exaggerated resistance responses to suprathreshold amounts of NA expressed as an increased steepness (about 135 %) of the resistance curve ($p < 0.005$)

5) Increased maximal strength of contraction expressed as a raised maximal pressor response (about 40 %) to supramaximal amounts of vasoconstrictor agents during constant flow conditions ($p < 0.001$)

Except for the first point which constitutes an interesting and at first sight unexpected exception these results agree well with earlier findings in other major systemic beds (Folkow *et al* 1970 a b) Thus also the perfused hindquarter preparation of SHR exhibits a steeper resistance curve and a higher maximal pressor response as compared to NCR while the threshold NA response is the same for NCR and SHR. These earlier results as well as the present ones from the renal vascular bed strongly suggest a morphological adaptation of the resistance vessels in SHR in such a way that they have strucurally adapted to the higher pressure load by increasing their wall thickness In perfusion studies on the entire systemic circulation thus including the kidneys (but for technical reasons not the coronaries) as well as on the above mentioned hindquarter preparation flow resistance was increased even at maximal dilatation in SHR as compared to NCR Combining the evidence there appears to be one factor common for all major systemic circuits namely an increased wall thickness of the resistance vessels but it is obvious that such a structural change may affect the lumen at maximal dilatation in different ways

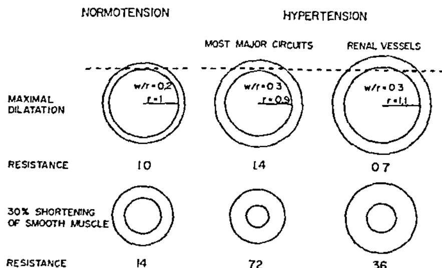


Fig 3 Schematic illustration of the hemodynamic effects of an increased wall/lumen ratio in the resistance vessels during hypertension (modified from Sivertsson 1970) indicating the difference between the renal resistance vessels and those of other major circuits when related to the resistance vessels in normotension

(Fig 3) Thus it appears as if the thickened wall then encroaches upon the lumen in most systemic vascular regions while it in the kidney for some reason (see below) is combined with increased lumina of the resistance vessels However once substantial smooth muscle contractions are induced resistance will also in the kidney be higher in SHR for any given level of smooth muscle shortening This series of studies illustrates the necessity of measuring also flow resistance at maximal dilatation to characterize the haemodynamics of a vascular bed as this minimal resistance forms the baseline from which active vascular changes start

Measurements of resting flow resistance in kidneys of SHR and NCR normally perfused with blood by the heart (Folkow *et al* unpublished observations suggest that resting vascular tone is then high enough to be at or beyond the crossing over point of the resistance curves in Fig 2 In case the renal resistance vessels in human essential hypertension display similar structural changes as those of the SHR the situation may be largely the same in human kidneys as in the blood perfused SHR kidneys Thus the repeatedly observed increase of renal flow resistance in hypertension (see Pickering 1968) may at last in many cases be explained by the fact that normal renal flow autoregulation has enhanced the tone of the preglomerular vessels so as to reach beyond the crossing-over point in the "resistance curves" as illustrated in Fig 2

The question arises why the resistance vessels of the SHR kidneys differ from those other major circuits with respect to the luminal dimensions during maximal dilatation when related to the situation in NCR The present results cannot provide

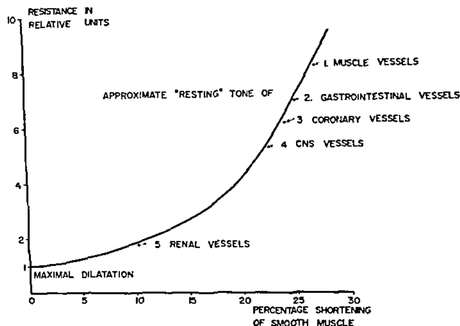


Fig 4 Approximate levels of 'resting' vascular tone in the major systemic circuits They are plotted along a simplified "unit resistance curve to illustrate more clearly the considerable differences between resistance ratios during "rest' and maximal dilatation

any definite answer to this problem, but an important functional difference exists between the renal vascular bed and those of most other tissues, which might be of relevance In the resting" steady state the tone of the resistance vessels is far higher in tissues like skeletal muscle, gastrointestinal tract, myocardium and central nervous system than in the kidneys This is evident from the far greater difference between the regional flow resistances during rest and maximal dilatation in the first mentioned circuits, as compared with the kidney (see *e g* Mellander and Johansson 1968) Expressed in another way, the average vascular tone is placed decidedly higher on the individual 'resistance curve" for each of the first mentioned vascular beds than is the case for the kidneys (see Fig 4) In this Figure the approximate levels of 'resting" vascular tone for each of the mentioned vascular beds, (which, of course in reality have their own characteristic resistance curves thanks to their highly different dimensional design) have been plotted along a simplified 'unit resistance curve to illustrate more directly the different relationships between flow resistance during rest and that during maximal dilatation Due to the differences in resting vascular tone, this being especially low in the kidneys and especially high in *e g* skeletal muscles it is evident that the structural adaptation of the resistance vessels is likely to be initiated from quite different levels of functionally determined wall/lumen ratios Possibly such a difference in functional equilibrium of wall/

lumen ratio leads to somewhat different types of morphological adaptation, with respect to the relationship between wall thickness and luminal dimensions with the result that the lumina become increased at maximal dilatation in the kidney but more or less decreased in the other tissues In any case, these results certainly make it highly unlikely that any structurally based Goldblatt mechanism should be of etiological reference in these animals

It appears as earlier discussed in detail (Folkow *et al* 1970 b, see also Sivertsson 1970) that only the presence of an increased contractile wall mass raising the wall lumen ratio can alone explain all the differences between NCR and SHR It appears, moreover, to be clear from many morphological studies, particularly from the very careful and convincing study by Furuyama (1962) that hypertension is associated with a considerable wall hypertrophy of precapillary resistance vessels particularly of their media when related to the luminal dimensions The question what 'comes first during the development of 'primary' hypertension the increased pressure or the morphological change in wall/lumen ratio, still remains unanswered but different possibilities have been discused at length in previous papers (*e g* Folkow *et al* 1970 a, b, Sivertsson 1970) Whichever the case, even if the adaptive structural changes of the resistance vessels should be secondary in nature as to their time course their early involvement and great importance for the establishment and further development of a sustained hypertensive state seems obvious from these earlier studies The common denominator for the systemic circuits appears to be an increased media thickness, which can evidently take place at the expense of either the inner radius, the outer radius or both (see Fig 3) depending perhaps on the regional level of basal tone and resulting in varying degrees of encroachment upon the lumen at maximal dilatation Howeved it has the important consequence that for given more significant levels of vascular smooth muscle activity resistance will always be higher in those resistance vessels that display an increased wall thickness

To summarize in analogy with earlier findings the present results suggest that a characteristic and hemodynamically important structural adaptation of the resistance vessels has taken place also in the SHR kidneys However the thickened vascular walls seem here to be combined with increased vascular lumina at maximal dilatation while in other circuits the walls seem to encroach upon the lumina It should be stressed however that this stduy was performed on relatively young animals (6—7 months) and the situation might in this very respect be a different one in older animals in case the normal morphological adaptation of the vessels gradually deteriorates into obliterative-degenerative vascular changes

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Effect of Amphotericin B on the Frog Skin in vitro. Evidence for Outward Active Potassium Transport across the Epithelium

By

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Abstract

NIELSEN R. *Effect of amphotericin B on the frog skin in vitro Evidence for outward active potassium transport across the epithelium* Acta physiol scand 1971 *83* 106—114

It is shown that amphotericin B (5×10^{-5} M) increases the permeability of the frog skin to potassium chloride and urea The potassium flux was more strongly increased from the inside to the outside than from the outside to the inside of the skin whereas the chloride and the urea fluxes were equally enhanced in both directions The increased potassium flux from the inside to the outside could not be explained by a potassium loss from the cells It is suggested that a part of the high outward potassium flux was due to an active outwards transport of potassium

The active transport across the amphibian skin and urinary bladder is generally thought to occur in two steps passive diffusion across the outer borders of the epithelium followed by active extrusion into the fluid bathing the inner borders (Koefoed Johnsen and Ussing 1958 Frazier and Leaf 1963) Koefoed Johnsen and Ussing (1958 suggest for the frog skin that the active mechanism is a sodium potassium exchange pump whereas Frazier and Leaf 1963 suggest for the toad bladder that the active mechanism is an electrogenic pump

The biological effects of the polyene antibiotics (e g Amphotericin B) have been attributed to an increased permeability of the cell membranes The increased permeability is probably due to an interaction of the polyene compound with the membrane bound sterols (Kinsky Luse and Van Deenen 1966) Lichstenstein and Leaf (1965) and Bently (1967 have shown that amphotericin B added to the mucosal side of the bladder stimulates the sodium transport The permeability of the bladder to small solutes e g chloride and thiourea is also greatly increased by amphotericin B (Lichstenstein and Leaf 1965

If the sodium pump mechanism is a sodium potassium exchange pump and amphotericin B increases the permeability of the outer cell membrane one might ex

pect that it would be possible to detect an active transport of potassium from the inside of the skin to the outside, when the outside was treated with amphotericin B

This paper presents results which indicate that there was an active transport of potassium from the inside to the outside in the isolated frog skin during amphotericin B treatment, although the potassium transport was smaller than the simultaneous inwards transport of sodium

Methods

The experiments were performed on male and female frogs (Rana temporaria) The frogs were kept partially immersed in tap water at about 4° The skin was dissected from pitched animals and divided into two symmetrical halves one used as control and the other for the experiment The skins were mounted in perspex chambers (area 7 cm³) and bathed in areated Ringer s solution (Na^+ = 115 0 K^+ = 2.5, Ca^{++} = 1 0 HCO_3 = 2 4, Cl = 117 1 mM pH = 8 2) The shortcircuit experiments were performed according to the method of Ussing and Zerahn (1951) with an automatic voltage clamp apparatus which could be programmed to disconnect the short circuit current every five minutes allowing the potential to be measured for 15 sec In all experiments where fluxes were measured the skins were short-circuited during the whole measuring period The fluxes are in some cases expressed in term of permeability coefficients as defined by Andersen and Ussing (1957)

$$P = (a_2V_2 - a_1V_1) \,/\, A \times a_5 \,(t_2 - t_1)$$

Here a_1 and a_2 designate the radioactivity originating from side II in one ml of solution from side I at times t_1 and t_2 respectively, V_1 and V_2 are corresponding volumes of the solution on side I A the area of the membrane and a_4 the mean activity in one ml of solution from side II during the period from t_1 to t_2 The pH osmolarity and the potassium concentration were measured routinely The potassium content of the Ringer s solution and tissues were determined w [illegible] The sodium 22 and potassium 42 were counted [illegible] and the chloride 36 and urea C-14 with a Pack [illegible] using 10 ml scintillation mixture (Bray 1960) [illegible] skin (7 cm²) was cut out of the [illegible] (wet weight) and dissolved in 5 [illegible] solution was then evaporated to d[illegible] were removed for assay of potassium 42 and total potassium The total amount of potassium 42 in the skins was determined by using a sample from the Ringer s solution to which the potas[illegible]

[illegible] was used as the water soluble preparation Each vial contains 50 mg lyophilized amphotericin B powder, 41 mg sodium deoxycholate 10 mg di sodium phosphate 0 89 mg monosodium phosphate and 6 2 mg *sodium chloride* the amphotericin diluent solution had the same composition except that the amphotericin B was omitted

Results

The effect of amphotericin B on the short circuit (SCC) and potential (PD) across the isolated frog skin

Fig 1 A shows the result of adding 5×10⁻⁵ M amphotericin B to the medium bathing the outside of the frog skin Fig 1 B shows the corresponding control to which was added the same amount of amphotericin B diluent as was present in the am

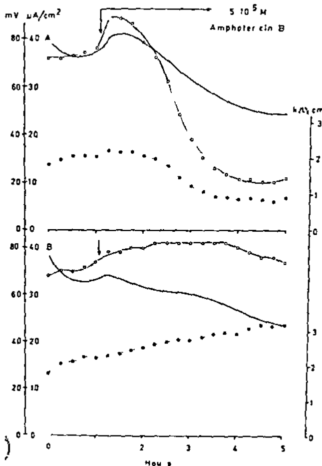


Fig 1 Effect of amphotericin B on the short circuit current and potential across the frog skin.

A — At the arrow amphotericin B was added to the outside of the frog skin to give a concentration of 5×10^{-5} M to the inside was added the same amount of amphotericin B diluent as present in the amphotericin B solution

B — Control skin half At the arrow the same amount of amphotericin B diluent as present in the amphotericin B solution was added

——— short-circuit current (μA/cm²)
○—○ potential (mV)
●—● resistance (ohm × cm²)

photericin B solution Within 1 to 5 min one observes the onset of a small increase in SCC and PD The increases in SCC were about 5—25 % when the experiments were performed in the period December—April During the summer the increase in SCC was normally of the same magnitude as in the control (0—10 %) 20—60 min after the addition of the amphotericin B the PD started to drop reaching its lowest value 1—3 hrs after the addition In December—April the PD decreased to 50—20 % of the value it had before the addition of the amphotericin B during the summer the decline in PD was frequently less pronounced In about 20 % of the experiments no effect on PD and SCC was observed The same concentration of amphotericin B (5×10^{-5} M) on the inside alone had no effect on SCC and PD

Effect on the sodium and potassium outflux

Five experiments were performed in which the sodium and potassium outflux were measured simultaneously on the same skin half It appears from Table I that both the sodium and the potassium outflux were increased (exp A) but the potassium

TABLE I Effect of amphotericin B on the sodium and potassium permeabilities from the inside to the outside of the frog skin

Exp no	P out for potassium $cm \times sec^{-1} \times 10^{-7}$			P out for sodium $cm \times sec^{-1} \times 10^{-7}$			P out K/Pout Na		
Period	A—B	B—C	C—D	A—B	B—C	C—D	A—B	B—C	C—D
1a	1 9	211	467	1 3	5 6	19 0	1 5	37 6	24 6
1b	4 2	6 2	6 3	2 1	2 1	1 9	2 0	3 0	3 3
2a	2 1	29 0	145	0 8	1 4	1 3	2 4	12 0	17 4
2b	1 6	2 8	2 1	1 4	1 2	1 4	1 1	2 3	1 6
3a	3 2	13 3	38 2	1 2	1 5	1 9	2 7	9 0	19 8
3b	4 5	6 4	7 8	1 0	1 3	1 4	4 4	4 8	5 8
4a	7 0	92 6	18 8	2 7	7 6	5 7	2 6	12 3	3 3
4b	6 2	9 0	11 4	2 7	3 2	3 8	2.2	2 7	3 0
5a	1 4	277	201	1 2	5 8	12 1	1 2	47 9	16 6
5b	1 7	3 7	5 0	1 1	1 6	1 7	1 6	2 3	2 9

outflux was increased much more than the sodium outflux From the control group (Table I exp B) it is seen that the amphotericin B diluent alone has a very limited effect on the fluxes

Effect on the permeability to chloride and urea

The effect of amphotericin B on the permeability of the frog skin to substances that are normally not actively transported across the skin, viz chloride and urea, was tested For both substances influx and outflux were measured The results were essentially the same for fluxes in either direction The addition of amphotericin B (Table II) results in an increase of about 2—3 times in the fluxes in the 1st 1 h period and

TABLE II Effect of amphotericin B on the permeability of the frog skin to urea and chloride

	Number of expts	Control ±S E	Amphotericin B ±S E	
Period		A—B	B—C	C—D
Urea	6	1 51±0 21	5 18±0 91	10 03±1 75
Chloride	6	1 78±0 50	2 97±0 47	7 31±0 84

In each experiment following 20 min of equilibration for the isotopes the permeability across the tissue was determined for one 1 hr control period (period A—B) Amphotericin B was then added to the outside of the skin to give a concentration of 5×10^{-6} M 10 min after the addition of the amphotericin B solution the permeability was determined for two further 1 hr periods (periods B—C and C—D)

Table III Effect of amphotericin B on the potassium influx and outflux

Exp no	Outflux µeq 7 cm²/hr				Influx µeq 7 cm²/hr			
Period	A—B	B—C	C—D	B—D	A—B	B—C	C—D	B—D
1	0 036	2 337	1 948	4 285	0 013	0 063	0 150	0 213
2	0 010	0 811	1 986	2 797	0 041	0 075	0 123	0 198
3	0 285	0 196	0 181	0 377	0 052	0 050	0 076	0 126
4	0 036	0 376	0 722	1 098	0 089	0 093	0 481	0 574
5	0 008	0 144	0 258	0 403	0 024	0 070	0 111	0 181
6		0 525	0 496	1 021		0 138	0 208	0 346
7		1 071	0 848	1 919		0 084	0 122	0 206
8		0 160	0 132	0 292		0 052	0 075	0 127
9		1 201	0 863	2 064		0 173	0 259	0 432
10		0 695	0 436	1 131		0 233	0 316	0 549
11		0 093	0 141	0 234		0 054	0 073	0 127

The potassium influx and outflux were measured simultaneously on symmetrical halves of a frog skin Period A—B is a 1 h control period Amphotericin B was added to the outside of the skin to give a concentration of 5×10^{-4} M 10 min after the addition of the amphotericin B the fluxes were determined for two further 1 hr periods (period B—C and C—D) Period B—D is the sum of periods B—C and C—D

of about 6 times in the following 1 h period Table I shows that amphotericin B had the same effect on the sodium outflux It therefore appears that amphotericin B had a rather nonspecific effect on the permeabilities

If the sodium and the potassium outfluxes were mainly extracellular the ratio of the permeability coefficients should be equal to the ratio of the mobilities—i e 1 5 for large pores) The fact that the ratio of the potassium -and the sodium permeabilities was much higher than 1 5 (Table I) indicates that a part of the potassium appears from a compartment with a high potassium concentration This compartment is probably the cells in the epithelium Accordingly most of the potassium outflux was cellular Thus amphotericin B produced a pathway for potassium in the outer membrane of the frog skin

Effect on potassium outflux and influx

Table III shows a series of experiments in which the potassium outflux and influx were measured separately on symmetrical skin halves In the 1 hr control period (A—B) there was no significant difference between the potassium influx and outflux However in the two 1 hr periods period B—C and C—D) after the addition of the amphotericin B solution the potassium outflux was always higher than the influx (2—40 times The calculated linear regression line between the outflux and the influx for the two hour period given in table III period B—D was Influx = 0 29—0 00013 Outflux (µeq) the correlation coefficient r = 0 067 was not significantly different from zero (0 90 > p > 0 80 Thus there was no correlation between the outflux and the influx In these experiments the skin was short-circuited and the osmolarity and the potassium concentration were the same on both sides of

TABLE IV Effect of amphotericin B on the potassium content in the isolated frog skin

Exp no	μeq potassium 42/7 cm^2 control	exp	potassium loss (μeq)	outflux μeq
column no	1	2	3	4
1	5 07	4 33	0 74	1 37
2	4 18	3 97	0 21	0 54
3	3 16	2 91	0 25	3 89
4	3 92	4 02	—0 10	0 88
5	4 29	4 19	0 10	2 55
6	5 75	4 23	1 52	3 41
7	5 62	5 01	0 61	1 18
8	4 82	4 35	0 47	0 33
9	4 90	4 07	0 83	1 15

In each experiment following about 80 min of equilibration for the isotope amphotericin B was added to the experimental skin half to give a concentration of 5×10^{-6} M The same amount of amphotericin B diluent as present in the amphotericin B solution was added to the control skin half 10 min after the addition of the amphotericin B the potassium outflux was determined for [illegible]

columns 1 and 2 is given in column 3

the skin Under these circumstances, the flux ratio Outflux/Influx should be equal to 1 for passive fluxes (Ussing 1949) The fact that the flux ratio for potassium was higher than 1 during amphotericin B treatment indicates an active transport of potassium from the inside to the outside of the frog skin However the possibility exists that a part of the potassium outflux was due to an irreversible loss of potassium from the cells to the outside solution (*e g* cell membranes bursting)

The effect of amphotericin B on the potassium content in the isolated frog skin

To investigate whether there was a potassium loss from the skin, a series of experiments was performed in which amphotericin B was added to one skin half and amphotericin B diluent to the other half (the control) The potassium outflux was measured for a 2 hr period The skin halves were then removed from the chamber and the amount of potassium-42 in the skins was measured The experiments in Table IV indicates that there might be a potassium loss from the cells ($0\,025 > p > 0\,01$) The percentage of labelling was the same in both skin halves during these conditions, namely 74 6 % ± SE 3 3 in the control and 73 3 ± SE 3 4 in the amphotericin B treated skin halves (n = 7)

Discussion

According to the two membrane hypothesis (Koefoed-Johnsen and Ussing 1958) the frog skin can be treated as composed of a "outward facing membrane" which is selectively permeable to sodium and permeable in a nonselective way to small anions

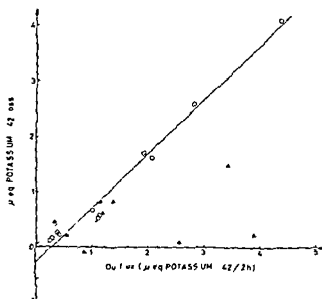


Fig 2 Open circles: minimal potassium-42 loss calculated from (potassium-42 outflux)—(potassium-42 influx) when it was assumed that the potassium flux across the skin was passive and all potassium-42 lost was lost to the outside solution. Continuous line: regression line for these points. Dashed lines: ±3 times the standard error of the estimate. *Triangles*: measured total potassium-42 loss plotted against the measured potassium-42 outflux.

like chloride. The inward facing membrane is permeable to potassium and small anions but impermeable to free sodium.

According to experiments carried out by Ussing and Windhager (1964) and Farquhar and Palade (1966) the outward facing membrane is located just beneath the cornified layer and the inward facing membrane is identical with the membrane limiting the extracellular space throughout the epithelial layer.

During the incubation of the skin in the absence of amphotericin B the epithelial cells exchange some of their potassium with potassium-42 in the inside solution (the inward facing membrane is permeable to potassium) but not with potassium-42 in the outside solution (the outward facing membrane is impermeable to potassium). The addition of amphotericin B to the Ringer's solution induces an increase in the potassium permeability of the outward facing membrane. Accordingly the high flux ratio found in Table III might be due to 1 an underestimation of the influx because the potassium-42 in the Ringer's solution to which the potassium-42 was added is supposed to be 100 % potassium-42 coming from the outside pile up in the skin when it exchanges with potassium present in the epithelial cells, 2 the outflux could be too high because the cells lose potassium-42 to the outside solution, 3 because there is an active outwards transport of potassium during these conditions.

In 4 expts the total amount of potassium in the skin which had exchanged with potassium-42 in the outside solution were measured after 3 hrs of incubation (1 hr without and 2 hrs with amphotericin B). This amount 0.12 μeq/7 cm² (range 0.0 – 0.20) indicates that 2.4 % of the total potassium in the skin had exchanged with the outside solution. This amount was too small to account for the high flux ratio found in Table III. Moreover the outflux must also be underestimated since

potassium-42 coming from the inside also exchanges with the potassium in the cells Thus the high outflux must be due to a potassium-42 loss from the cells or to an active transport of potassium

If the potassium-42 movement across the skin were passive, we can get an estimate of the minimal potassium loss necessary to account for the flux ratio observed in Table III by subtracting the influx from the outflux This is a minimal estimate since it requires all potassium-42 lost is lost to the outside solution The minimal potassium 42 loss (K 42 loss min) as function of the potassium-42 outflux measured for the two hour period given in Table III (period B—D) is plotted in Fig 2 (circles) The linear regression line (Fig 2 continues line) is given by

(K-42 loss min) = $-0\,28 +$ (K-42 outflux), in μeq

The standard error of the estimate for the estimating equation was 0 106 μeq and correlation coefficient r = 0 996 From the data in Table IV it appears that during incubation with potassium-42 on the inside, the total amount of potassium-42 in the skin was 4 63 ± 0 28 μeq (SE) By sectioning the skin paralel to the outside surface Hansen and Zerahn (1964) has shown that less than 50 % of the total potassium was localized in the epithelial layer, the rest being localized in the con nective tissue Koefoed Johnsen (cited by Ussing 1965) has shown that the potassium in the connective tissue and the epithelial layer exchange nearly with the same rate Therefore only about 2 μeq potassium-42 was present in the epithelial layer From Fig 2 it appears that in order to explain the high potassium-42 outflux observed in some skins, it was necessary to postulate that more potassium 42 than present in the whole epithelial layer had been lost to the outside solution

An estimate of the maximal (total) potassium-42 loss from the skin which took place during amphotericin B treatment is given in Table IV The maximal loss as function of the total potassium 42 outflux during 2 hrs incubation with amphotericin B is plotted on Fig 2 (triangles) From this it appears that when the outflux was higher than 2 μeq/2 hrs the measured maximal loss (triangels) was less than the minimal possible loss (circles)

Thus it is very unlikely that the potassium loss could account for the flux ratio found Therefore we suggest that a part of the high outflux of potassium-42 was due to an active outwards transport of potassium

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Release of Noradrenaline from the Dog Heart In Situ after Intravenous and Intracoronary Administration of 5-Hydroxytryptamine

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Abstract

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The following experiments have been carried out in order to establish the validity of the hypothesis that the cardiac stimulatory effects of 5 hydroxytryptamine (5-HT) are mediated by catecholamines Release of cardiac noradrenaline (NA) has been observed after administration of 5 HT either intravenously or intracoronarily After labelling the heart with ^{3}H 5 HT, it was possible to release this amine by cardioaccelerator nerve stimulation even after treatment with an adrenergic β blocker It is concluded that the cardiac stimulatory effects of 5 HT are mediated by NA, this NA is mainly derived from sympathetic nerve endings by intraneuronal exchange for 5 HT

Using isolated rabbit atria, Levy and Michel-Ber (1956), found that tachyphylaxis to the stimulatory effects of 5-hydroxytryptamine (5-HT) could be abolished by adrenaline and that these effects were blocked by ephedrine On the basis of these observations, they proposed that the stimulatory effects of 5 HT were mediated by catecholamines Jacob and Poite Bevierre (1960) and Trendelenburg (1960) showed that the effects of 5-HT on isolated rabbit heart were diminished by pretreating the animal with reserpine in addition, Trendelenburg (1960) established that they were inhibited by dichloroisoproterenol More recently, (Jacob and Fillion 1965 1967) it has been shown on the dog heart *in vivo* that the cardiac stimulatory effects of 5 HT are not a consequence of a reflex stimulation, since ganglionic blocking agents failed to decrease them, and that they could be inhibited by adrenergic β blockers or by reserpinisation Moreover, using bioassay determinations an increase in the noradrenaline (NA) level in the coronary venous blood was observed after administration of 5-HT

In the present study the hypothesis that the cardiac stimulatory effects of 5 HT are mediated by catecholamines has been investigated using biochemical assay

techniques NA has been identified and assayed spectrophotofluorometrically in the coronary venous blood after intravenous injections of 5-HT. In addition 5-HT has been injected into the coronary arteries after labelling the cardiac catecholamines by infusion of tritiated NA, and the release of radioactivity into the coronary venous blood has been measured

The possible uptake and release of 5-HT within the sympathetic nerve endings have been investigated by measuring the outflow of radioactive material on stimulation of the sympathetic cardiac nerves after labelling the heart with ^{3}H-5-HT

Methods and material

Mongrel dogs of either sex weighing 8 to 20 kg were anesthetized with sodium pentobarbital (30 mg/kg) or with a mixture of chloralose and urethane They received atropine (2 mg/kg i.v.) in order to avoid the parasympathetic effects of 5-HT, and a ganglionic blocking agent, chlorisondamine (2 mg/kg) to eliminate the reflex components of the response to 5-HT The dogs were ventilated artificially with room-air through a tracheal cannula A right or left thoracotomy was performed and after heparinization the coronary sinus was cannulated via the right atrial appendage In the first series of experiments (i.v. administration of 5-HT) the coronary sinus was connected to the jugular vein using a polyethylene tube A side arm permitted collection of samples of coronary sinus blood In other experiments (intracoronary injections) the circumflex artery was cannulated close to its origin at the aorta, using polyethylene tubing and supplied with blood from the femoral artery through a silicone-oil filled drop counter (Lindgren 1958) The intracoronary injections were performed using a side arm to the polyethylene tubing The coronary blood flow was recorded throughout the experiment thus permitting quantitative estimations of the outflow of radioactivity from the heart and, at the same time giving an indication of the functional state of the preparation The arterial pressure was recorded from the femoral or the carotid artery and the heart rate was measured from the arterial pressure pulse by means of an ordinate writer After section of the cardio-accelerator nerves just distal to the stellate ganglion the postganglionic fibres were stimulated supramaximally for 30 sec (2 msec duration 10 to 20 cps) using a Grass stimulator All the recordings were made on a Grass 5 B polygraph

Spectrophotofluorimetry

20 ml samples of blood from the vena cava and the coronary sinus were collected simultaneously once before and after the injection of 5-HT Blood plasma was separated by centrifugation The fluorimetric method of Anton and Sayre (1962) was used to determine catecholamines The mean recovery for the standards was 70 ± 6 % and this has been corrected for in our results

Radioisotopic assay

The coronary circumflex artery was perfused during 1 hr with 250 μCi of ^{3}H-NA (rate of infusion 2 to 5 [illegible] tissue) A period of at least one hour was allowed between the end of the perfusion and the start of the 5-HT administration to allow the washout of most of the extraneuronal ^{3}H-NA from the heart As Axelrod *et al* (1961) reported for the rat heart the disappearance of ^{3}H-NA from the dog heart took place in two phases (see Fig 1) 5-HT was injected mainly during the second phase corresponding to the release of intraneuronally bound ^{3}H-NA (Iversen 1965) However because the preparation can only be maintained in optimal condition for a few hours in some experiments the 5-HT injections were started before the first phase had quite [illegible] completion Blood samples (2 ml) were collected simultaneously from the coronary sinus and the vena cava every 10 min during the experiment During the study of the effects of 5-HT or stimulation of the cardioaccelerator nerves (6–8 trials for each experiment) the blood was continuously collected from both sites In experiments using ^{3}H-5-HT the same procedure was followed but 500 μCi ^{3}H-5-HT were perfused through the circumflex artery in lieu of ^{3}H-NA

After centrifugation of the blood samples the plasmas were isolated and 0.5 ml aliquots taken for determination of radioactivity Generally the activity was between 3000 and [illegible]

Fig 1 Outflow of radioactivity in the coronary sinus blood after perfusion of the left coronary artery with 250 μCi of ^{3}H NA during one hour The zero time corresponds to the end of the perfusion The radioactive level decreases in two phases the first (rapid) phase passes within 1 1/2 to 2 hrs the second (slow) phase is then apparent

cpm/ml of blood The scintillator solution (15 ml for each sample) consisted of 0 4 % 2 5 di phenyloxazole and 0 01 % 1 4 bis 2 (4 methyl 5 phenyloxazoyl) benzene in a mixture of equal volumes of toluene and ethyleneglycol monoethylether Corrections have been made for back ground activity ($\simeq$22 cpm) but not for quenching Since the counting conditions were always similar and we were primarily interested in changes in the levels of radioactivity it was not necessary to determine the absolute activity

Measurements of the radioactivity were performed using a Tricarb liquid scintillation spectrometer (Packard Instrument Co Inc La Grange, Ill, U S A)

Chromatography

40 to 60 ml blood samples were collected in centrifuge tubes on ice with 0 1 % EDTA and ... e the plasma the volume ... ecipitation of the plasma ... d the remainder of the ... mm × 3.5 cm) of Dowex ... was washed successively with 20 ml of water +0 1 % EDTA 15 ml of phosphate buffer pH 6 5+0 1 % EDTA and 10 ml of distilled water The washes were bulked and counted These effluents and washes ... nn was ... ir 3-0- ... 7 and ... acuum and dissolved in ethylacetate and counted This corresponds to the 4 hydroxy 3 methoxy phenylglycol (MOPEG) fraction The water phase was adjusted to pH 1 to 2 and extracted with ether and ethylacetate After evaporation to dryness and redissolving in ethylacetate the organic phase containing the 4-hydroxy 3 methoxy mandelic acid (VMA) fraction was counted The aqueous phase which contained conjugated MOPEG and VMA was counted

Materials

^{3}H DL-NA (specific activity 8 76 C/mM) was obtained from New England Nuclear Boston Mass U.S A ^{3}H 5 HT (as creatinine sulphate specific activity 500 mCi/mM) was obtained from The Radiochemical Centre Amersham England We thank CIBA Produkter A.B Valingby Sweden for the gift of chlorisondamine (Ecolid) The other chemicals were obtained from normal commercial sources

Results

1 Release of NA into coronary venous blood after i v administration of 5 HT

In all 5 dogs used in these experiments an increase in NA concentration in the coronary sinus blood was observed after injection of 5 HT into the vena cava

Fig 2 shows a dose response curve obtained in one of the experiments A dose range of 25 to 200 μg/kg has been studied (all doses of 5 HT refer to the quantity

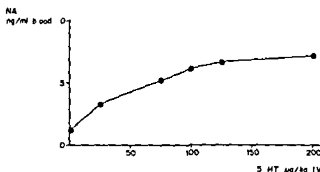


Fig 2 Concentration of NA in the coronary sinus blood following intravenous administration of increasing doses of 5 HT (in 1 dog)

of 5 HT creatinine sulphate injected) In the control coronary blood the NA concentration was 1 2 ng/ml A maximal concentration of 7 2 ng/ml was obtained after administration of 200 µg 5 HT/kg i v The coronary sinus blood flow changed from 40 ml/min in the control to 160 ml/min thus the total outflow of NA per minute increased from control values of 48 ng/min to ca 1 µg/min

Fig 3 summarizes the results obtained in the 5 dogs using 100 µg 5 HT/kg iv The NA concentration in coronary sinus blood (mean ± S E) increased from 0 81 ± 0 06 ng/ml to 5 45 ± 0 35 ng/ml A simultaneous increase in NA concentration from 0 70 ± 0 04 ng/ml to 3 05 ± 0 81 ng/ml was observed in the vena cava blood The means of the NA concentration values in coronary sinus (12 determinations) and in the vena cava (9 determinations) were significantly different ($P < 0.02$)

Regarding the coronary sinus flow the calculated amount of catecholamines appearing in the coronary sinus increased from 0 035 ± 0 01 µg/min in the control blood to 0 508 + 269 µg/min after 100 µg 5 HT/kg i v

2 Release of radioactivity in the coronary venous blood after intracoronary injections of 5 HT in H NA labelled heart

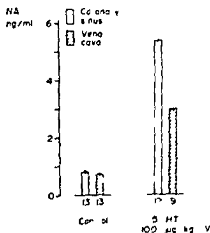


Fig 3 Concentration of NA in coronary sinus blood (open bars) and in vena cava blood (hatched bars) collected simultaneously before (control) and after iv injections of 100 µg kg 5-HT The figures below the bars indicate the number of determinations the experiments were carried out in 5 dogs

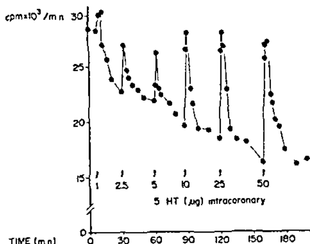


Fig 4 Release of radioactive NA from the dog heart *in situ* after intracoronary administration of 5-HT The first 5 HT administration was performed 90 min after the end of the perfusion of 250 µCi of ^{3}H NA The numbers below the arrows refer to the doses of 5 HT

These experiments were performed on 15 dogs in which the left coronary (circumflex or anterior descending) artery was cannulated After perfusion of this artery with ^{3}H NA, the release of radioactivity in the coronary sinus blood was measured following intracoronary administration of 5 HT The doses used ranged from 1 to 300 µg corresponding to ca 0 02 to 6 µg/g of tissue

In 12 dogs a significant increase in the outflow of radioactivity in the coronary venous blood followed the administration of 5 HT in a dose range of 1 to 200 µg In 2 of these dogs doses of 50 and 100 ng of 5 HT were tested and still a significant increase in radioactivity was observed Fig 4 shows an experiment in which doses from 1 to 50 µg of 5 HT were administered into the circumflex artery An abrupt increase in radioactivity occurred in the coronary sinus blood 30 to 60 seconds after each administration of 5 HT The release was dose dependent between 1 and 50 µg increasing from 2000 to 11 000 cpm/min

The radioactivity was measured simultaneously in the vena cava and in the coronary sinus blood Initially the radioactivity in the vena cava was lower than in the coronary sinus blood but it increased progressively during the experiment so that the levels were the same after 3 to 5 hrs Evidently there was a build up of radioactivity in the general circulation during the experiment due to the release of radioactive material from the heart The marked increase in radioactivity observed in coronary sinus blood after intracoronary injection of up to 50 µg of 5 HT was not accompanied by a corresponding significant change in the radioactivity level in the vena cava blood This is illustrated in Fig 5 which shows an experiment in which a dose of 50 µg of 5 HT was administered into the circumflex artery Following administration of higher doses an increase in radioactivity level was consistently observed also in the vena cava blood simultaneously with or slightly (30 to 60 sec) after that observed in the coronary venous blood This increase however never

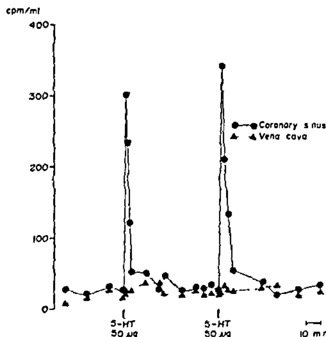


Fig 5 Radioactivity changes in the coronary sinus and the vena cava bloods in one dog injected intracoronarily with 50 µg 5-HT The first injection was performed 90 min after the end of the infusion of 250 µCi of ³H NA The interval between the injections is 1 hr

represented more than 20 % of the increase of radioactivity in the coronary sinus blood

3 Identification of the radioactive material released after administration of 5 HT

Chromatographic studies (2 expts) showed that the radioactivity of the control blood was present mostly (at least 95 %) as ³H-NA metabolites namely 4 hydroxy 3-methoxy-phenylglycol and 4-hydroxy-3 methoxy-mandelic acid After administration of 5-HT (50 to 100 µg intracoronary) the increase in radioactivity found in the coronary sinus was due mainly to the appearance of amines (NA or normetanephrine

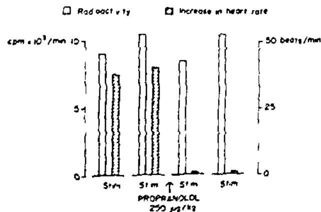


Fig 6 Release of radioactive 5 HT following nerve stimulation of the ³H 5 HT labelled dog heart in situ (500 µCi ³H 5-HT infused) The left cardioaccelerator nerves were stimulated supramaximally for 30 sec 2 ms duration 10 cps) The interval between each stimulation was 5 min The open bars represent the total radioactivity released after each stimulation expressed in cpm/min The hatched bars represent the increase in heart rate induced by the nerve stimulation expressed in beats per min The arrow indicates the injection of propranolol (250 µg/kg

4 Uptake and release of ³H-5 HT in the heart

In order to investigate if 5 HT was taken up by sympathetic nerve endings the cardioaccelerator nerves were stimulated after labelling the heart by perfusion with ³H 5 HT via the circumflex artery (4 dogs) An increase of the radioactivity in the coronary venous blood was observed after each stimulation one such experiment is shown in Fig 6

In order to minimise a possible release of 5 HT secondary to increased cardiac contractile activity induced by nerve stimulation an adrenergic β blocker (propranolol 0 25 mg/kg) was administred in 2 of the dogs With this dose the chronotropic and inotropic effects during nerve stimulation were markedly reduced (86 and 88 % respectively) but the increase of radioactivity was undiminished

Discussion

The present experiments support the hypothesis of Levy and Michel Ber (1956) that the cardiac stimulatory effects of 5 HT are mediated by NA An increase in NA concentration is observed in coronary sinus blood After intravenous injection of 5 HT a concomitant increase in NA concentration is also observed in the vena cava blood However since the NA concentration increases more markedly in the coronary sinus blood part of the NA released after 5 HT administration is of cardiac origin On the other hand the observed increase in NA concentration in vena cava blood is of such a magnitude that it cannot be explained unless it is assumed that a release of NA occurs also in the periphery from sources other than the heart This is supported by the fact that catecholamines are released from the adrenals of the cat after 5 HT administration (Reid 1952) The lungs are known to remove 5 HT from the circulation very rapidly Thomas and Vane (1967) claimed that 80 % to 98 % of the intravenously injected amount of 5 HT in the dog is removed by one passage through the lungs whereas Davis (1968) showed that in humans the corresponding removal is 30 to 60 % In any case since the total coronary flow represents only 3 to 5 % of the cardiac output (Rodbard *et al* 1953) the release of cardiac NA into the coronary venous blood must be induced by only a small percentage of the intravenously injected amount of 5 HT Most of the 5 HT not retained by the lungs will reach the periphery

The cardiac NA released by 5 HT probably originates from the nerve endings It has been shown by elaborate denervation techniques that in the dog and cat virtually all the cardiac NA is contained in postganglionic fibres (Cooper 1966) A participation of chromaffin like cells cannot be completely excluded but the existence of such cells is in fact not firmly established Jacobowitz (1967) described them in the cat rat guinea pig and mice hearts Truex (1950) found them in the dog heart but Dahlstrom (1965) could not demonstrate their existence in the same preparation Moreover it seems likely that even if these cells are present in canine cardiac tissue they are not numerous enough to account for the quantities of NA released in the present experiment moreover they contain mostly adrenaline (Euler 1963)

It has been shown in numerous studies that sympathetic nerve terminals can take up i v infused NA In particular, Chidsey and Harrison (1963) showed on the dog that, after a single i v injection of ^{3}H NA it is possible to release the labelled catecholamine by stimulation of the cardioaccelerator nerves Since 5 HT was administered during the second phase of disappearance of ^{3}H-NA, it can be assumed that the ^{3}H NA released by 5 HT originates from the nerve terminals The release of ^{3}H-NA observed after stimulation of the cardioaccelerator nerves in the same experiments supports this assumption

After labelling the heart by infusing ^{3}H-5 HT, it is possible to release it by stimulation of the cardioaccelerator nerves This release can be demonstrated even after treatment with an adrenergic β blocker which minimizes the possible release of 5 HT due to the increase in cardiac contractile activity induced by the nerve stimulation. Thus it is reasonable to assume that 5 HT enters the nerve terminals and releases NA from its storage sites Quantitative studies cannot be performed on the present preparation, but an exchange of 5-HT for NA probably takes place at the granule stores *This assumption is supported by the studies of Burgen and Iversen (1965) who* demonstrated a certain affinity of 5 HT for the sites of NA storage by measuring its ability to produce an inhibition of the NA uptake in isolated rat heart It is also in agreement with the results of Gillis (1964) who showed that subcellular fractions of rabbit heart are able to concentrate 5-HT to the same extent as NA and those of Tissari *et al* (1969) who showed that the model transport system proposed for accumulation of NA into isolated nerve endings is also applicable to 5 HT

In conclusion it seems that the cardiac stimulatory effects of 5-HT are mediated by NA The NA released by 5 HT is mainly derived from the sympathetic nerve endings where an intraneuronal exchange for 5 HT takes place

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It has been shown in numerous studies that sympathetic nerve terminals can tal up i v infused NA In particular, Chidsey and Harrison (1963) showed on the d that after a single i v injection of ^{3}H-NA, it is possible to release the labelled cat cholamine by stimulation of the cardioaccelerator nerves Since 5 HT was ac ministered during the second phase of disappearance of ^{3}H-NA, it can be assume that the ^{3}H-NA released by 5 HT originates from the nerve terminals The relea of ^{3}H NA observed after stimulation of the cardioaccelerator nerves in the same e periments supports this assumption

After labelling the heart by infusing ^{3}H-5 HT, it is possible to release it by stim lation of the cardioaccelerator nerves This release can be demonstrated even aft treatment with an adrenergic β blocker which minimizes the possible release of 5-H due to the increase in cardiac contractile activity induced by the nerve stimulatio Thus it is reasonable to assume that 5-HT enters the nerve terminals and releas NA from its storage sites Quantitative studies cannot be performed on the prese preparation but an exchange of 5-HT for NA probably takes place at the granu stores This assumption is supported by the studies of Burgen and Iversen (1965) wh demonstrated a certain affinity of 5 HT for the sites of NA storage by measuring i ability to produce an inhibition of the NA uptake in isolated rat heart It is also i agreement with the results of Gillis (1964) who showed that subcellular fractio of rabbit heart are able to concentrate 5-HT to the same extent as NA and those Tissari *et al* (1969) who showed that the model transport system proposed for ac cumulation of NA into isolated nerve endings is also applicable to 5 HT

In conclusion it seems that the cardiac stimulatory effects of 5 HT are mediate by NA The NA released by 5 HT is mainly derived from the sympathetic nerv endings where an intraneuronal exchange for 5 HT takes place

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discharge rate in sympathetic nerve fibres to peripheral vessels and a markedly increased release of catecholamines from the adrenal medulla into the circulation (Celander 1954 Watts and Westfall 1964) The blood catecholamine concentration which prevail during hypotension might either in itself or together with other factors related to the hypotension syndrome influence the behaviour of the thrombocytes *The aim of the present investigation was to evaluate any role of catecholamines* released from the adrenal medulla in the PVR increase which develops during hemorrhagic hypotension

Methods

Animals Cats, weighing 3 to 4.5 kg were used They were anesthetized by i p injections (30 mg/kg) of sodium pentobarbitone (Nembutal® Abbott)

All animals were exposed to a period of standardized hemorrhagic hypotension In one *group of 6 cats the adrenal glands were acutely denervated before exposing the animals to* blood loss This group will in the following be called *the denervated group* The animals in this first group were compared to 8 animals in a second group with intact adrenal innervation This group will be referred to as *the control group* Some results from this group has been reported in a previous work (Bo and Hognestad 1971) A third group of 7 animals also had their adrenals denervated before they were exposed to the hemorrhage However they received a continuous and standardized infusion of catecholamines during the period of hemorrhagic hypotension

Adrenal denervation The left adrenal gland was surgically isolated through an abdominal incision Only the vascular supply was left intact Both the nervous and vascular supply to the right adrenal gland were cut By this procedure the release of catecholamines was blocked *whereas the release of corticosteroids could continue (from the left gland)*

Ventilation After performing tracheostomy a muscle relaxant Alloferin (1/2 mg/kg) was given and positive pressure ventilation started with a piston pump respirator (The Ideal Respiration Pump C F Palmer Ltd London) The respiration frequency was 14 per min End tidal pressures were usually kept at 6 to 8 (inspiratory and 2 expiratory) cm of water respectivel by use of water seals The respirator s tidal volume was adjusted so as to keep the arterial P_{CO_2} at 30 mm Hg which is the normal level in the cat Carefully standardized hyperinflations of the lungs were carried out twice per hour The ratio of the respirator s tidal volume to the inspiratory peak pressure was calculated as an expression of the pulmonary compliance

Surgical procedures Pressure and flow recordings The animals were placed on a heated table *and covered by a polyethylene tent into which was led warm moist air* The animal s temperature was thereby maintained at the normal level

The thorax was opened widely by a sternum-splitting incision Catheters of polyethylene were introduced into the femoral artery the pulmonary artery and the left atrium for recordings of the femoral arterial pressure (P_{FA} — with a Statham P23Gb transducer) the pulmonary arterial pressure (P_{PA} — with a Statham P23Db transducer) respectively Catheters were also placed into the femoral veins for infusions

A flow probe was placed around the ascending aorta, and flow was recorded by a Nycotron square wave flowmeter (type 377 Nycotron A/S Norway) Pressure and flow transducers were connected to a six channel Sanborn recorder (Model 320 Sanborn Co California)

Mean pulmonary vascular resistance PVR *was calculated using the following simple* equation

$$PVR = \frac{P_{PA} - P_{LA}\ (\text{mm Hg})}{\text{Mean flow (ml/sec)}}$$

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pH [illegible] were done once per hour with Radiometer equipment (pH meter 22 equipped with a pH-electrode type G 297/G and a gas mixing apparatus) (Radiometer Copenhagen The arterial P_{CO_2} was calculated by the equilibration method using Siggaard Andersen s nomogram

Hematocrit values were determined by *means of an International microcapillary centrifuge* model M D

Induction of hemorrhagic hypotension Heparin, 500 I U /kg was given i v All the groups of animals were then bled in the course of 20 to 30 min through a catheter in the femoral artery into a plastic syringe The femoral arterial pressure, P_{FA}, was in all animals kept at a level of 45 to 50 mm Hg throughout the 3 h following the hemorrhage After one to two h of hypotension small retransfusions of blood were needed to maintain the arterial pressure at this chosen level

Infusions Harvard infusion pump (model 947, Harvard Apparatus Co, Mass) was used for the infusions Adrenaline or noradrenaline was infused at a rate of 5 µg/kg/min. Infusions of a mixture of the 2 hormones, consisting of 4 parts of noradrenaline and 1 part of adrenaline, were carried out at the same rate

Statistical analysis The Wilcoxon two-sample test was used for determination of statistical significance

Stock solutions of the drugs used

Nembutal®, Abbott, 60 mg/ml

Alloferin®, Roche, 5 mg/ml

Heparin®, AL, 1 mg/ml

Adrenalin®, AL, 1 mg/ml

Nor-exadrin Conc®, "Astra", 1 mg/ml

Results

A striking difference was observed between the changes in pulmonary arterial pressure (P_{PA}) in the group of denervated animals on the one hand and in the group of control animals on the other (Fig 1) Both groups showed an initial fall in P_{PA} down to approximately 70 per cent of the pre-hemorrhage level In the denervated group, however, the pulmonary arterial pressure remained at a relatively stable low level throughout the observation period whereas in the eight animals of the control group a secondary gradual and marked rise in that pressure was seen After 3 h the difference in P_{PA} between the 2 groups was highly significant ($P<0.01$)

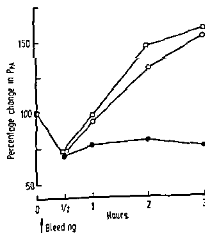


Fig 1 Development of mean pulmonary arterial pressure P_{PA}, during a 3 h period of hemorrhagic hypotension in cats
● mean values from 6 animals with denervated adrenals
○ mean values from 8 control animals with intact adrenal innervation
□ mean values from 3 animals with denervated adrenals and where an infusion of catecholamine (5 µg/kg/min 4/5 noradrenaline 1/5 adrenaline) had been carried out throughout the 3 h hypotensive period (see Methods)
Standard errors of the mean values amounted to ±6 per cent or less in the control group as well as in the denervated group

TABLE I Effect of 3 h hemorrhagic hypotension on level of circulating thrombocytes, pulmonary compliance, hematocrit, arterial pH and PVR in variously treated animals

Treatment of animals in various groups before and during hemorrhagic period (n = number of animals in each group)	Volume of blood withdrawn (in ml/kg)	Mean percentage fall in thrombocytes after 3 h	Mean percentage fall in 'compliance' after 3 h	Mean percentage rise in hematocrit after 3 h	Mean fall in arterial pH 3 h after bleeding	Mean percentage rise in PVR 3 h after bleeding
Denervation of adrenals (n = 6)	24 3	20	4 (rise)	0 5	0 29	83
No special treatments (Control group) (n = 8)	23 2	37	24	5	0 30	355
Denervated adrenals Adrenaline infusion during hemorrhagic period (n = 2)	26 7	28	6	0	0 50	225
Denervated adrenals Noradrenaline infusion during hemorrhagic period (n = 2)	26 4	16	10	10	0 53	270
Denervated adrenals Infusion of mixture of noradrenaline (4/5) and adrenaline (1/5) during hemorrhagic period (n = 3)	24 2	36	29	24	0 50	630

The average volume bled was the same in the two groups (Table I) The changes on left atrial pressure caused by the bleeding was also the same in the 2 groups of animals Here a gradual fall from about 5 mm Hg to between 2 and 4 mm Hg was found

The mean pulmonary vascular resistance (PVR) in the denervated group increased by 95 per cent of the prehemorrhage level during the observation period whereas in the control group a maximal mean rise of 350 per cent was found in the same period The difference between the two groups after 3 h was highly significant ($P<0.01$) (Fig 2)

Continuous infusions of adrenaline, noradrenaline or a mixture of these two hormones were then carried out in 7 animals with denervated adrenals Adrenaline (5 μg/kg/min) was continuously infused to 2 such animals, noradrenaline to 2 other animals, and the mixture of 4 parts of noradrenaline and 1 part of adrenaline to 3 further animals The infusions started simultaneously with the bleeding and was continued throughout the 3 h observation period In these animals the P_{PA} and PVR rose gradually and above the levels found in the denervated group In animals which received infusions of adrenaline and in those which received noradrenaline, the PVR values did not reach the levels seen in the control group (Table I) How-

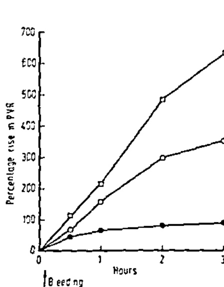


Fig 2

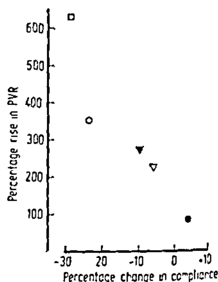


Fig 3

Fig 2 Perecentage rise in pulmonary vascular resistance, PVR during a 3 h period hemorrhagic hypotension in cats
● mean values from 6 animals with denervated adrenals
○ mean values from 8 control animals with intact adrenal innervation
□ mean values in 3 animals with denervated adrenals where an infusion of catecholamin (5 μg/kg/min 4/5 noradrenaline, 1/5 adrenaline) had been carried but during the 3 hypotension period
Standard errors of the mean values amounted to ±33 per cent or less in the control grou and to ±18 per cent or less in the denervated group

Fig 3 Correlation between the mean rise in pulmonary vascular resistance PVR and the mea change in pulmonary compliance after 3 h of hemorrhagic hypotension in groups of various treated cats
● group with denervated adrenals
○ control group
▽ group with denervated adrenals infusion of adrenaline during 3 h period
▼ group with denervated adrenals infusion of noradrenaline during 3 h period
□ group with denervated adrenals infusion of catecholamine mixture (4/5 of noradrenalin 1/5 adrenaline during 3 h period
For all infusions amounts of catecholamines given were 5 μg/kg/min

ever, infusions of the mixture of noradrenaline and adrenaline resulted in a P rise, which was somewhat more marked than that seen in the control group (Fig 1) Also a greater flow reduction was observed in these 3 animals Consequently the calculated mean PVR rose up to a level corresponding to 600 per cent of th initial value, a much more marked rise than seen in the control animals (Fig 2)

The number of thrombocytes in arterial blood fell in all animals during the perio of hemorrhagic hypotension (Table I) In the denervated group the thrombocyte were reduced by 20 per cent during the first hour of hemorrhagic hypotension N further reduction was observed to occur later in the experimental period Th animals in the control group showed a continuous fall in circulating thrombocyte during the 3 h of hypotension After this time period the mean thrombocyte numbe

was 37 per cent below starting level At 3 h the percentage fall in the number of thrombocytes was significantly different in the 2 groups ($P<0.02$) The animals in the infusion groups behaved similarly in this respect to the animals in the control group

All the animals also showed a fall in the hematocrit 1 h after bleeding Thereafter the hematocrit rose gardually to or above the prebleeding level In the denervated group the prebleeding hematocrit value and the value after 3 h were 37 5 and 37 per cent respectively In the control group the corresponding values were 37 and 39 per cent (Table I) The 5 animals in the infusion group, which had received noradrenaline, alone or mixed with adrenaline, showed a somewhat more pronounced rise in hematocrit with a 10 to 14 per cent rise in this parameter after 3 h (Table I)

The pulmonary compliance, calculated as the ratio of the respirator's tidal volume to the peak inspiratory pressure, rose by about 4 per cent in the denervated group, whereas it fell by about 24 per cent in the control group ($P<0.01$) (Table I) The animals which received catecholamine infusions behaved similarly to those in the control group

A fall in arterial pH was also observed This fall must have been due to metabolic acidosis, since arterial P_{CO_2} was kept normal and constant The control group and the denervated group showed the same fall in pH of about 0 3 units during the 3 h hypotension period, whereas the animals receiving catecholamine infusion showed a somewhat greater fall of about 0 5 units (Table I)

Discussion

The constriction of resistance and capacitance vessels and also the transvascular fluid shifts observed during increased sympathetic activity are mainly the result of an increased discharge rate in sympathetic vasomotor nerve fibres The circulating catecholamines appear to play a less important role for these vasomotor effects (Celander 1954) The markedly increased level of circulating catecholamines in blood during hemorrhage is mainly the result of release from the adrenal medulla (Watts and Westfall 1964) This reflex induced release can be abolished or reduced both by adrenal denervation (Malmejac 1964, Higashi 1966) and by epidural procain infusion (Watts 1965) In the present experiments an increased release of catechol amines was eliminated by adrenal denervation without interfering with the peripheral sympathetic vasoconstrictor effects of hemorrhage The cats in the denervated group tolerated a standardized, large blood loss just as well as did the animals in the control group (Table I) This is an indication of peripheral vasoconstriction being unimpaired in the animals with denervated adrenals

The results presented show that denervation of the adrenals abolished the marked and progressive pulmonary vascular pressure responses otherwise caused by hemorrhagic hypotension Consequently the rise in calculated PVR was small in the denervated group The small rise which did occur in this group may in part be explained as a passive rise in resistance secondary to the reduction in pulmonary blood

flow (Bo and Hognestad 1971) However, the large and progressive rise in PVR in the control group must be due to other factors These other factors appear to be drastically interfered with when the adrenals are denervated

A large output of catecholamines from normally functioning adrenals is thus an apparently important factor in the development of pulmonary hypertension during hemorrhagic shock The question arises then as to how this effect of the catecholamines is brought about. Do they act directly upon the pulmonary resistance vessels or are their main role to propagate thrombocyte aggregation with release of vasoactive substances from the thrombocytes and with increase in pulmonary vascular resistance as a secondary event? The fact that the marked pulmonary hypertension subsequent to a bleeding does not develop in thrombocytopenic animals with intact adrenal innervation (Bo and Hognestad 1971) indicates that a role of this latter type is the more important one

Plasma concentrations of catecholamines in man and animals during hemorrhagic hypotension have been found to be in the range of 20 to 100 $\mu g/l$ (Rosenberg *et al* 1961 Watts and Westfall 1964) Both *in vitro* studies and experiments on living animals have shown that adrenaline may promote the aggregation of thrombocytes (Haslam 1967 Stehbens 1969) When adenosine diphosphate (ADP) has been added to human platelet rich plasma in concentrations too small to induce aggregation and adrenaline is subsequently added to give a final concentration of 5 10^{-7} M (≈ 90 $\mu g/L$) irreversible thrombocyte aggregation takes place (Mills and Roberts 1967) However addition of adrenaline alone to the same concentration did not cause aggregation *In vitro* aggregation induced by collagen and thrombin is similarly potentiated by the rather small concentration of adrenaline of 10^{-8} M (≈ 2 $\mu g/L$) (Thomas 1968) It is reasonable to suggest therefore that the high levels of circulating catecholamines during hemorrhage may condition or influence the thrombocytes towards aggregation and other types of alterations The smaller fall seen in circulating thrombocytes in the group of animals with denervated adrenals also indicates that this may be the case

Catecholamines in the concentrations found in blood during hemorrhage will probably not by themselves induce thrombocyte aggregation Cofactors which could be important for such an effect are *e g* collagen from traumatized tissue (Thomas 1968) free fatty acids (Shore and Alpers 1963) or factors from traumatized red cell (Bennett 1968) such as for instance ADP The plasma levels of free fatty acids have been found to be greatly increased during shock (Warner 1969) Further investigations on the detailed mechanisms leading to thrombocyte alterations during hemorrhagic hypotension is obviously needed

The adrenal medulla releases a mixture of adrenaline and noradrenaline During hypotension in cats mainly noradrenaline is released (Euler and Folkow 1953) By stimulation of all nerve fibres to both adrenal glands of the cat at a stimulation frequency of 10 imp/sec a release of catecholamines of about 5 $\mu g/kg/min$ was found to take place (Celander 1954) This can probably be regarded as the maximal physiological rate of release and was therefore mimicked with the present infusions

to the hypotensive animals with denervated adrenals In these experiments no difference could be found between the PVR responses obtained in animals receiving adrenaline and in those receiving noradrenaline The 1 4 mixture of the 2 catecholamines, however, gave PVR increases which were much more marked (Fig 2) We have no ready explanation for this phenomenon but it is interesting to note that this mixture presumably mimics the output from the animal s own glands

A rough estimate of pulmonary compliance was obtained by observing the ratio of the respirator s tidal volume to the peak inspiratory pressure employed The ratio showed a moderate decrease during the hypotensive period in the control animals This alteration in compliance was abolished in the denervated group, but restored in parallel with the increase in pulmonary vascular resistance in animals with denervated adrenals, where catecholamines were infused during hypotension The close correlation between the rise in PVR and the change in compliance after 3 h (Table I and Fig 3) makes it reasonable to explain the fall in compliance as an event secondary to the rise in PVR Pulmonary congestion and perivascular edema could possibly be factors behind the altered compliance

The present experiments allow the conclusion that catecholamines released from the adrenal medulla during hemorrhagic hypotension are essential for the development of the markedly increased pulmonary vascular resistance seen The mechanisms whereby the catecholamines are involved in this development appear to include alterations in the circulating thrombocytes (Bo and Hognestad 1971)

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Ullevål Hospital, Norway

Diameter and Elasticity of the Ascending Aorta during Infusion of Noradrenaline

By

H Aars

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Abstract

Aars, H *Diameter and elasticity of the ascending aorta during infusion of noradrenaline* Acta physiol scand 1971 *83* 133—138

[illegible] usually found in dogs It is concluded that at comparable pressures contraction of aortic smooth muscle tissue induced by infusion of noradrenaline produces a small but significant constriction of the ascending aorta, and at low pressures only, increases dynamic stiffness of the vessel

Until recently, the effects of contraction of aorta could be studied only in isolated aortic strips or tubes (McDonald 1960 Bader 1963) It has therefore been difficult to ascertain the part played by smooth muscle in the *in vivo* regulation of dimension and elasticity of the thoracic aorta Results differed, even after techniques were introduced for measuring the diameter of the intact aorta Some investigators observed that contraction of the smooth muscle tissue by infusion of noradrenaline led to constriction and stiffening of the aortic wall (Peterson *et al* 1960 Barnett *et al* 1961), others found no such effects (Remington 1962) The discrepancies were probably due to the smallness of the response, and to interference with smooth muscle tone in the control state It therefore became important to avoid thoracotomy and exposure of the vessel

Pieper and Paul (1969) studied the pressure/diameter relationship of thoracic aorta in dogs by means of a catheter tip strain gauge inserted through the femoral artery They found that at comparable pressures hemorrhage caused a reduction

in aortic diameter, but minor or no changes in the modulus of elasticity or the calculated pulse-wave velocity Similar results were obtained for the ascending aorta in rabbits by intravenous infusion of noradrenaline (Aars 1971) The diameter of ascending aorta was measured inside the intact thorax as the transit time of ultrasonic pulses between two small crystals glued to the aortic wall 1—2 weeks earlier Whereas this technique was adequate for comparing aortic diameters in normal and hypertensive rabbits (Aars 1969), the stiffening effect of the glue, and localized toxic necrosis of smooth muscle cells may have reduced the effects of smooth muscle contraction In particular, impediment of the pulsatory movements of the wall would have been expected This could explain why the pressure strain elastic modulus was found to be so much higher than reported by others (Aars 1969)

In the present investigation of aortic response to noradrenaline the technique for measuring aortic diameter has been improved Each crystal, instead of being glued to the vessel was fastened to the ascending aorta with 2 minute stitches in adventitia and outer part of media A quickly-formed layer of loose connective tissue helped to stabilize contact with the vessel and the crystals never adhered to or affected the median coat of the artery

Material and Methods

Experiments were performed in 18 rabbits anesthetized with 3 ml 1 % chloralose and 3 ml 25 % urethane per kg The animals received half the amount i v and the rest i p If necessary more urethane was given in the course of the experiments The animals were tracheotomized but respiration was not assisted Arterial blood pressure was measured in the right common carotid artery 1—3 cm above the aortic arch, through a catheter connected to a Statham transducer (P23Gb) Diameter of the ascending aorta, about 5 mm proximal to the brachiocephalic trunk was measured as the transit time of ultrasonic pulses between two piezo electric crystals (Aars 1969) The crystals (about 2 × 2 mm in size) were sutured to the aortic wall with 5—0 silk on an atraumatic needle 3—15 days earlier Pressure and diameter were recorded on a Sanborn recorder at a paper speed of 100 mm/s

The relationship between diameter and pressure was studied at various blood pressure levels, ... ne ... ch ... nt ... un

after the initial pressor response When several tests were performed on the same animal the interval between the end of one run with noradrenaline and the next control run was at least 20 min usually more Aortic pressure and diameter were read for two pulse beats at each pressure level and mean diastolic and peak systolic values were found

The influence of noradrenaline was studied as its effect on diastolic aortic diameter and on the pulsatile diameter variations The first follows directly from the relationship between pressure and diameter in late diastole but the second more dynamic aspect was examined by calculating the pressure train elastic modulus (E_p) and the theoretical pulse wave velocity (PWV) The equation for E_p is

$$E_p = \frac{\Delta P \times D \times 1333}{\Delta D} \text{ dynes/cm}^2$$

where Δ D (mm) is the increase above diastolic diameter D produced by the pulse pressure Δ P (mm Hg) and 1333 is the conversion factor for mm Hg to dynes (Peterson Jensen and Parnell 1960) According to the Moens Korteweg equation, the relationship between PWV and the stiffness of the arterial wall is

$$\text{PWV} = c = \sqrt{\frac{E_p}{2\rho}} \text{ m/s}$$

where ρ is the density of blood, 1 055 (Patel *et al* 1963)

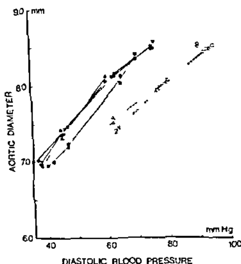


Fig 1

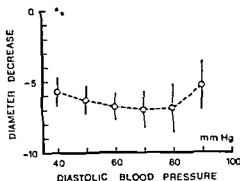


Fig 2

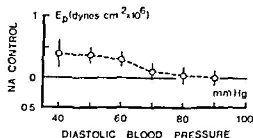
Fig 1 Relationship between blood pressure and diameter of ascending aorta before (solid symbols) and during (open symbols) i v infusion of noradrenaline (4 μg/kg min) Four sets of experiments (sequence ● ▲ ■ ▼) in one animal with intervals of 20—75 min. Blood pressure changed by alterations of blood volume

Fig 2 Reduction of diastolic aortic diameter during infusion of noradrenaline (4—6 μg/kg min) in per cent of diameter at the same blood pressure during control runs Mean $\pm$2 SE from 31 sets of experiments in 18 rabbits Blood pressure changed by alterations of blood volume

Results

Infusion of noradrenaline produced a reduction in diastolic aortic diameter at all blood pressures as can be seen from Fig 1, which shows repeated sets of experiments in one rabbit In this animal the decrease was about 7 % and control diameters were reached at about 20 mm Hg higher pressures during infusion of noradrenaline Mean reduction of diastolic aortic diameter in 31 experiments on 18 animals was 5 3—7 0 % of control diastolic diameter, with a mean for all pressures of 6 6 % (Fig 2)

Average pressure strain elastic modulus (E_p) before infusion of noradrenaline ranged from about $0\,5\times10^6$ to 2×10^6 dynes/cm² (Fig 3, lower part) With noradrenaline there was a small but significant increase in E_p at 40 50 and 60 mm Hg i e 67 50 and 30 % respectively, but no change at higher levels (Fig 3 upper part) PWV calculated for mean values of E_p were from 5 2 m/s (40 mm Hg to 9 9 m/s (90 mm Hg) Corresponding figures during infusion of noradrenaline were 6 8 m/s and 9 9 m/s respectively Mean heart rate, which in control runs was 262 beats/min, did not change consistently with noradrenaline



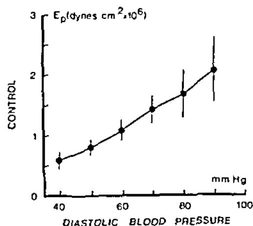


Fig 3 Effect of noradrenaline (4—6 μg/k ... preceding control run *Lower part* E_p in control runs

Discussion

Present results confirm that in rabbits noradrenaline at comparable blood pressure will reduce the diameter of the ascending aorta by about 6 % (Aars 1971) This is similar to results from the descending aorta of dogs (Barnett *et al* 1961) but contrary to the lack of effect observed by Remington (1962) Peterson *et al* (1960) described a decrease in aortic diameter but gave no information concerning the pressure/diameter relationship of descending aorta before and during iv infusion of noradrenaline Pieper and Paul (1969) studied the aortic response to removal of up to 20 % of estimated blood volume in 40 dogs Diastolic diameter of descending aorta was usually reduced at comparable pressures during hypovolemia presumably due to increased muscular activity of the aorta Results were presented from only nine selected animals however and based on these data the average decrease of diastolic aortic diameter at 40 and 90 mm Hg may be calculated as 0 8 % and 3 1 % respectively — considerably less than at the same pressures during noradrenaline infusion in the present study

The aortic response to noradrenaline may also be evaluated by effects on aortic distensibility, which is defined as the slope of the pressure/diameter curves (P/D mean or diastolic values) As before (Aars 1971) the decrease in aortic diameter tended to be less at 40 mm Hg and 90 mm Hg than at intermediate pressures This would suggest that distensibility was reduced at the lowest pressures and increased at

the highest However, the differences were small, and for all practical purposes aortic distensibility was generally unaffected by noradrenaline at or below control blood pressures The same conclusion can be drawn from studies in dogs (Barnett *et al* 1961)

Control values of E_p were about 1/4 of those earlier described for ascending aorta of rabbits (Aars 1969), presumably because the crystals no longer affected the aortic wall Even so, E_p — and theoretical PWV — was slightly higher than usually found in the dog (Patel *et al* 1963, Remington 1963, Gow and Taylor 1968 Pieper and Paul 1969) In theory, "dynamic" stiffness of the arterial wall will increase with increasing pulse frequency (McDonald 1960), but experimental evidence exists only for isolated vessels (Bergel 1961) The difference in the findings between rabbits and dogs might therefore be explained by the higher pulse frequency in rabbits Other species differences, or a higher pulse pressure in the common carotid artery than in the ascending aorta, could also be involved

The "dynamic" stiffness of the aortic wall was increased by noradrenaline, but only at very low pressures This is contrary to earlier studies in rabbits (Aars 1971) and in dogs (Barnett *et al* 1961) For rabbits, the present response of E_p probably reflected technical improvements Peterson *et al* (1960) claimed that E_p of descending aorta in dogs could be raised by i v infusion of noradrenaline, but no data for E_p *versus pressure were presented in support of this statement* Barnett *et al* (1961) found that noradrenaline (20—80 μg/min to dogs weighing 13—18 kg) increased the dynamic stiffness of thoracic aorta at mean pressures from 40 to 160 mm Hg but the increase was largely independent of blood pressure At pressures where in rabbits, E_p was increased by 30—67 %, it rose by an average of 11—13 % in aorta *of dogs (calculated from values of dynamic extensibility index $\Delta D/\Delta P$* (Barnett *et al* 1961)) As the doses of noradrenaline were about equal, the discrepancy in the results could lie in higher control tone of the smooth muscle tissue in the exposed aorta, and in higher resting control blood pressures in the dogs In that event the results would indicate that an increase of smooth muscle tone raises the dynamic *stiffness of aorta only below resting control* pressures, and that this effect may disappear, even if the constrictive effect on mean or diastolic aortic diameter is maintained

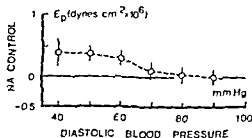
This work was supported by Anders Jahre s Fund for the Promotion of Science the Norwegian Council on Cardiovascular Diseases the Norwegian Research Council for Science and the *Humanities and J L Tiedemanns Tobaksfabrik* Joh H. Andresens Medical Fund

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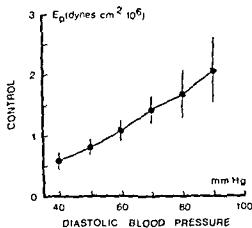


Fig 3 Effect of noradrenaline (4—6 µg/k min on aortic pressure strain elastic modu

trol runs

Discussion

Present results confirm that in rabbits noradrenaline at comparable blood pressur will reduce the diameter of the ascending aorta by about 6 % (Aars 1971) This similar to results from the descending aorta of dogs (Barnett *et al* 1961), but con trary to the lack of effect observed by Remington (1962) Peterson *et al* (1960 described a decrease in aortic diameter but gave no information concerning th pressure/diameter relationship of descending aorta before and during iv infusion o noradrenaline Pieper and Paul (1969) studied the aortic response to removal of u to 20 % of estimated blood volume in 40 dogs Diastolic diameter of descendin aorta was usually reduced at comparable pressures during hypovolemia presumabl due to increased muscular activity of the aorta Results were presented from onl nine selected animals however and based on these data the average decrease o diastolic aortic diameter at 40 and 90 mm Hg may be calculated as 0 8 % and 3 1 % respectively — considerably less than at the same pressures during noradrenalin infusion in the present study

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From the Departments of Physiology and Biophysics University of Göteborg and University of Lund, Sweden

On the Distribution of Sodium in the Rat Portal Vein

By

NILS BÅTH, BORJE JOHANSSON and OLOF JONSSON

The ionic composition of smooth muscle is a matter of much confusion which also makes understanding of its electrophysiology difficult The problem is particularly complex with regard to the distribution of sodium as the large Na^+ amount in the voluminous extracellular space complicates measurement of the smaller fractions in other tissue compartments

Vascular smooth muscle is especially rich in NA and a significant amount of 'bound Na appears to exist in the arterial wall (*e g* Friedman and Friedman 1967) Studying the ^{24}Na uptake in isolated rat portal veins Haljamae *et al* (1970) found also here a fraction of such "excess Na' If the cell membranes of the veins were damaged by freezing and thawing a homogenous fluid phase was obtained in the tissue as indicated by the fact that the ^{14}C sucrose space then corresponded almost to total tissue water (76 6 and 79 7 ml/100 g w w , respectively) whereas in intact portal veins it is 49 3 ml/100 g w w *i e* some 62 per cent of the total tissue water After 2 h incubation in normal Krebs the ^{24}Na space of the damaged muscles increased to 93 1 ml/100 g w w , showing that they were capable of holding even more Na than could be accounted for by the fluid phase supposed to be in equilibrium with the surrounding medium The tentative interpretation was that the fraction of the ^{24}Na space exceeding total tissue H_2O was now bound' to the damaged tissue

The aim of the present investigation was to characterize this excess sodium in more detail by examining the ^{24}Na uptake at different Na^+ concentrations in the medium Experiments were performed on intact as well as frozen and thawed preparations of portal vein

The general schedule of the uptake experiments with regard to dissection preincubation freezing thawing and incubation as well as *the methods for* determining tissue water and distribution volumes of radioactive tracers have been described previously (Arvill et al 1969 Haljamae *et al* 1970) The total $[Na^+]$ of the incubation media containing ^{24}Na was varied by replacing NaCl with sucrose in the standard *Krebs solution* on an osmotic bas s or by adding NaCl Incubation media with subnormal $[Na^+]$ were thus isotonic but those with supra normal $[Na^+]$ were hypertonic Uptake periods of 60 min were used

The results are summarized in Fig 1 The $[Na^+]$ of the incubation media are represented on the abscissa and the total tissue content of exchangeable Na as calculated from the experimentally determined ^{24}Na space and $[Na^+]_o$ on the ordinate The open circles represent the total Na content of *frozen and thawed* preparations The second curve from above (x symbols) was obtained by multiplying their total

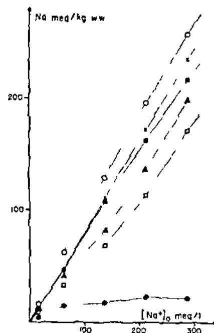


Fig 1 Sodium content (meq/kg w w) in isolated portal veins as a function of $[Na^+]_0$ Ordinate values calculated from determinations of ^{24}Na space after 60 min incubation o—o Total Na content in preparations damaged by freezing and thawing, x—x The Na content that these preparations would have shown if Na had only been dissolved in their tissue water equilibrated with medium, ●—● Difference between the former two curves, ▲—▲ Total Na content in "intact" portal veins, ■—■ The Na content that these preparations would have shown if their entire tissue fluid had equilibrated with medium, □—□ The Na content of the extracellular space (^{14}C sucrose space) of the 'intact' portal veins

tissue water with $[Na^+]_0$ and illustrates, therefore, the Na prevailing in these damaged preparations, had they contained no other Na compartments but the homogenous fluid phase equilibrated with the medium The difference between these two curves represents "excess Na" and is plotted separately (closed circles) If this reflects binding of Na to a homogenous population of anionic sites these would show 50 per cent saturation at an $[Na^+]_0$ of about 40 meq/l

The fourth curve from above (triangles) illustrates the Na content of *intact'* portal vein preparations incubated at different $[Na^+]_0$ If their total tissue water had contained the same Na concentration as the medium the curve represented by the filled squares would have been obtained At supranormal $[Na^+]_0$ this curve deviates slightly from the corresponding one calculated for frozen and thawed muscles (x-symbols) The reason is that intact preparations had a relatively lower water content after hypertonic incubation whereas the damaged ones showed no such changes The curves for intact muscles (triangles and filled squares) show that the ^{24}Na space is less than total tissue water, except at the lowest value of $[Na^+]_0$ (15 meq/l) where the points overlap At higher $[Na^+]_0$ the curves diverge to reach a maximal difference around the $[Na^+]$ of normal Krebs after which they converge again

Thus, whereas the damaged preparations showed an ability to accumulate more Na than could be accounted for by the total tissue water, the intact ones kept the Na concentration of some fluid compartment below that of the medium The open squares in Fig 1 represent the Na content of the "extracellular space in intact muscles ($[Na^+]_0$ multiplied by the ^{14}C sucrose space) In a two compartment tissue model the difference between total Na (triangles) and extracellular Na (open

squares) would be looked upon as the Na content of intracellular fluid For normal Krebs the data of Fig 1 would then give an intracellular Na of 12 3 meq/kg w w or 43 2 meq/l intracellular water

However, if the Na binding found in the damaged preparations operates also in intact muscles $[Na^+]_i$ cannot be calculated on the basis of a two-compartment model If binding sites were all extracellular, intact muscles would bind as much Na as the damaged ones but bound Na (filled circles) would then in normal Krebs exceed the difference between total (triangles) and extracellular free Na^+ (open squares) leaving no Na for the intracellular fluid Therefore, some of the binding sites which in the damaged muscles are exposed to the Na^+ of the medium are not so in intact ones Instead they must be in contact with that fluid phase of the intact tissue which has a lower $[Na^+]$ than the medium. The total amount of *bound* Na in intact preparations cannot however, be determined from these results, as the relative distribution of extra- and intracellular binding sites exposed to different Na concentrations is not known

It is clear that determination of $[Na^+]$ in vascular smooth muscle from data of this kind is practically impossible Depending on how one balances the Na-"binding" function revealed by uptake in damaged preparations, and the Na restricting function shown by intact muscles one can obtain any value for $[Na^+]_i$ between 0 and 43 meq/l at incubation in normal Krebs Estimation of $[Na]_i$ from the ordinate intercept of the slow phase of wash-out curves, obtained after loading the tissue with ^{24}Na might be a better method, despite the difficulties associated with the curve analysis This approach was recently used for portal vein resulting in an $[Na^+]_i$ value of 13 1 meq/l in normal Krebs (Jonsson 1971) This corresponds to 3 5 meq/kg w w The present data for total and free extracellular Na would then give a bound Na fraction of 8 8 meq/kg w w for intact portal vein in normal Krebs

The Na distribution in the intact portal vein remains uncertain, but the present analysis suggests the following qualitative compartmentalization of exchangeable sodium 1 an extracellular fluid in equilibrium with the [Na] of the surrounding medium 2 an intracellular fluid with low $[Na]^+$, about 13 meq/l, 3 and 4 bound Na which is located both extra and intracellularly The reduction of total H_2O in intact compared to frozen and thawed muscles in hyperosmotic solution (closed squares compared to x symbols) indicates an osmotic action of NaCl on cell volume even after 1 h incubation

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Acta physiol. scand 1971. 83. 142—144
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Mechanism of Noradrenaline Hyperpolarization in Spinal Cord Motoneurones of the Cat

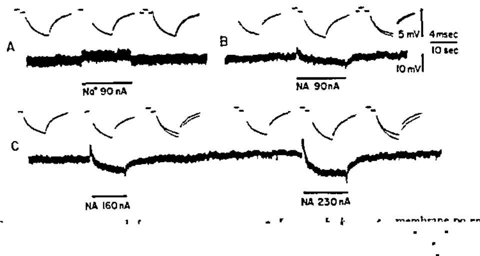
By

I Engberg and K C Marshall

The depressant effect of iontophoretically applied noradrenaline (NA) on spinal motoneurones has been shown to be related to a hyperpolarization of the neuronal membrane (Curtis 1968, Phillis, Tebecis and York 1968, Engberg and Thaller 1970) It has not been possible to demonstrate any increase in membrane conductance in relation to this hyperpolarization and the possibility of an active ion transport generating the potential shift has been discussed However signs of a small membrane conductance decrease have been reported (Engberg Marshall and Sonnhof 1971) In order to confirm these findings we have in the present work measured membrane conductance with a more accurate technique involving the use of shielded recording electrodes (Eide and Engberg, to be published)

The parallel effects of NA on membrane potential and membrane conductance of a motoneurone are illustrated in Fig 1 Three different doses of NA were ejected extracellularly by iontophoresis evoking increasing amounts of hyperpolarization of the cell (records B and C) The magnitude of the membrane conductance decrease during the NA ejections is best demonstrated in the superimposed records to the right in each series and can be seen to increase with the dose in parallel with the hyperpolarization The control application of sodium ions (record A) shows that the changes were not related to the current passage The conductance changes observed in most cells were quite small only occasionally larger than those illustated but always in the direction of a decrease

Upon the establishment of the fact that NA hyperpolarization in motoneurones is accompanied by a membrane conductance decrease the question arises of a causal relationship between these phenomena Membrane potential changes caused by ionic conductance changes can be expected to be dependent on the initial membrane potential This is indeed the case with the NA hyperpolarizations in motoneurones their amplitudes were regularly found decreased during moderate conditioning depolarization and increased during hyperpolarization without there being any related changes in the membrane conductance decrease Strong conditioning depolarization performed in a few cells with the aid of double intracellular electrodes even caused a reversal of the NA hyperpolarization into a depolarizing response Fig 2 A shows



the electrode resistance has been subtracted and 16 pulses averaged electronically in each record The first record in each set of three was taken immediately before the corresponding drug ejection the second record during the maximal drug action the third record consists of the two others superimposed

the control response at near normal membrane potential B the response during a period when the cell was kept continuously depolarized by an intracellular current injection of 70 nA and C shows the augmented NA hyperpolarization recorded shortly afterwards during the passage of hyperpolarizing current Accurate measurements of the membrane potential level at which the NA effect is reversed have so far proved difficult because of the changes in electrode coupling resistance that take place when large currents are passed Some indication of the level is given by the observation that more depolarizing current was needed to reverse the monosynaptic EPSPs than the NA responses (*cf* Fig 2 B) although the respective locations of the two types of synapses in relation to the polarizing electrode have to be taken into account in this comparison

The present finding of a voltage dependent and reversible hyperpolarization in conjunction with a conductance decrease is compatible with a mechanism by which the hyperpolarization is generated by a decreased passive ion flux Neuronal hyperpolarizations and membrane conductance decreases caused by NA have been found also in cerebellar Purkinje cells by Siggins *et al* (1971) These authors have reported that no reversal of the NA hyperpolarization is found in the membrane potential range of −10 to −80 mV However their finding in a few cells of a greater response during artificial hyperpolarization is in accordance with the present results

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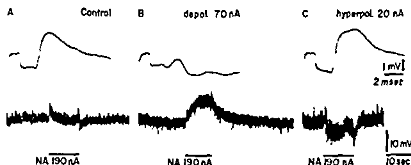


Fig. 2. *Lower traces*: Membrane potential, *A*, shortly after impalement, showing moderate hyperpolarizing effect of NA ejected as indicated by bar, *B*, during continuous artificial depolarization of the cell by intracellular injection of current, showing reversed action of NA. *C*, during hyperpolarization, showing large hyperpolarizing NA response. The absolute membrane potential levels in the records are not shown. *Upper traces*: Averaged monosynaptic EPSPs evoked from the homonymous muscle nerve and recorded immediately before the corresponding NA ejections. The EPSP in *B* is strongly reduced but not reversed by the depolarizing current; in *C* it is slightly increased. The admixture of a small IPSP is revealed in *B* and *C*.

cell membrane as does the NA response. Further, the slow EPSPs in sympathetic ganglia have recently been found to be generated by an ionic conductance decrease (Weight and Votava 1970) as have the acetylcholine depolarizations in cortical neurones (Krnjević, Pumain and Renaud 1971).

This work was supported by the Swedish Medical Research Council (B71-14X-735-03A). K.C.M. is the holder of a Killam Postdoctoral Fellowship from the University of British Columbia.

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Acta physiol. scand. 1971. 83. 145—149

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Effect of Angiotensin on Plasma-Free Fatty Acids in Dogs

By

STEIN AKRE and OLE DANBOLT MJOS

Received 5 February 1971

Abstract

AKRE, S and O D MJOS *Effect of angiotensin on plasma free fatty acids in dogs* Acta physiol scand 1971 *83* 145—149

tissue

Evidence is accumulating that angiotensin induces a fall in plasma free fatty acids (FFA) Rothlin *et al* 1962, Nakano and Kusakari 1963, Heidenreich *et al* 1964, Kaley *et al* 1967, Iizuka *et al* 1970) The exact mechanism of this response is unknown, although several hypotheses have been proposed Thus, a cholinergic-mediated action has been indicated by Iizuka *et al* (1970), who found that atropine blocked the depressive effect of angiotensin on plasma-FFA As sympathetic nerve activity is known to have a lipolytic effect (Rosell 1966), the response to angiotensin might result from reflex inhibition of sympathetic activity due to a rise in arterial blood pressure Furthermore, angiotensin might reduce adipose tissue blood flow (FBF) thereby lowering net release of FFA, as shown for sympathetic nerve stimulation (Rosell 1966) Inhibition of lipolysis by angiotensin locally in adipose tissue is also possible. In the present study, the contribution of these mechanisms to the plasma FFA lowering effect of angiotensin has been studied in anesthetized dogs

Materials and methods

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direct recorder Arterial plasma FFA were measured according to Dole (1956), as modified by Trout *et al* (1960)

The effects of angiotensin were studied in two series of experiments In the first series changes in concentration of plasma FFA were examined, the second series was designed to study blood flow in the isolated inguinal fat pad

The *first series* consisted of 11 expts in dogs pretreated with atropine (0.5 mg/kg i.v.

further increased (to about 0 4 μg/kg min) to reach the same $\overline{AP}$ as that achieved by angiotensin 0 2 μg/kg min, before ganglionic blockade At each dose, blood for FFA measurement

cannulated, and the venous blood from the fat pad led through a drop-counter and retransfused to the animal via a jugular vein This procedure allowed continuous recordings of blood flow (FBF) which were performed before and during i v infusion of angiotensin (0 2 μg/kg min In 4 of the animals, recording of FBF was also performed before and during angiotensin infusion after ganglionic blockade with pentolinium tartrate The doses of angiotensin tested were the same as in the first series of experiments during ganglionic blockade, 0 2 and 0 4 μg/kg min, respectively Stable FBF was usually reached after 10 min infusion at each dose.

Results

First series Plasma FFA was unaffected by atropine administration Infusion of angiotensin after atropinization reduced plasma FFA both before and after β adrenergic receptor blockade (Table I) At an infusion rate of 0 2 μg/kg min the average reduction was 36 % before and 30 % after propranolol treatment In two animals receiving higher doses of angiotensin (0 4 μg/kg min), the reduction was 50 and 56 % respectively However, when examined 45 min after stopping the infusion of angiotensin, plasma FFA exceeded control values by an average of 15 %, this over shoot was not seen in animals treated with propranolol Ganglionic blockade produced a fall in arterial blood pressure Angiotensin raised blood pressure to control level and induced an average fall of 18 % in plasma FFA A further increase in the angiotensin dose (to about 0 4 μg/kg min) to reach the same pressures as obtained by 0 2 μg/kg min of angiotensin before blockade caused plasma FFA to return to control concentrations

Second series Average blood flow in the isolated inguinal fat pad measured 3 6 ml/min. Angiotensin (0 2 μg/kg min, i v) reduced FBF by an average of 36 % ($p < 0 01$) (Table II) After ganglionic blockade, and hence lowering of arterial pressure, 0 2 μg/kg min of angiotensin had no effect on FBF However when blood pressure was raised with higher doses to about the same level as with angiotensin before blockade angiotensin increased FBF by an average of 28 % ($p < 0 001$) Angiotensin produced reductions in plasma FFA were measured in two dogs and found to be of the same magnitude as in the first series of experiments from 1225 μEq/l to 815 and from 725 μEq/l to 525, respectively Concomitantly, angiotensin

TABLE I Arterial concentrations of FFA (μEq/l) in control state and during i v infusion of angiotensin before and after β-adrenergic receptor blockade or ganglionic blockade

Dog number	Control	Angiotensin 0.2 μg/kg min	Propranolol Control	Propranolol Angiotensin 0.2 μg/kg min	Pentolinium tartrate Control	Pentolinium tartrate Angiotensin 0.2 μg/kg min	Pentolinium tartrate Angiotensin 0.4 μg/kg min
1	364	243	182	122			
2	516	387	188	188			
3	267	200	219	63			
4	375	250	344	281			
5*	625	375	518	241			
6*	759	310	531	219			
7	353	412			353	294	412
8	1071	761			571	380	428
9	512	333			384	333	435
10	848	238			333	286	324
11	643	—			476	452	476
Mean	576	351	330	186	424	349	415
±S E	71	51	66	33	44	31	25
P	<0.025		<0.05			<0.02	
Mean $\overline{AP}$ (mm Hg)	142	200	140	200	99	148	193
Mean HR (beats/min)	129	133	92	107	117	118	118

* Angiotensin infused at 0.4 μg/kg min

TABLE II Fatty tissue blood flow (FBF) (ml/min) in control state and during i v infusion of angiotensin before and after ganglionic blockade

Dog number	Control	Angiotensin 0.2 μg/kg min	Pentolinium tartrate Control	Pentolinium tartrate Angiotensin 0.2 μg/kg min	Pentolinium tartrate Angiotensin 0.4 μg/kg min
12	1.9	0.1	2.0	2.0	2.6
13	2.7	2.3	1.3	2.7	3.4
14	4.5	2.5	2.0	2.0	3.0
15	3.4	3.4	3.3	3.0	3.8
16	9.0	6.3			
17	1.6	0.8			
18	2.1	1.0			
Mean	3.6	2.3	2.2	2.5	3.2
±S E	1.0	0.8	0.1	0.3	0.3
P	<0.01			<0.001	
Mean $\overline{AP}$ (mm Hg)	113	149	66	118	140

reduced net release of FFA from adipose tissue in the first dog from 0.66 to 0.53 μEq/min, while in the second dog a net FFA release of 0.13 μEq/min changed to a net FFA-uptake of 0.66 μEq/min with angiotensin

Discussion

A fall in plasma FFA could be due either to reduced net release of FFA from adipose tissue or to increased uptake of FFA in other tissues In myocard, uptake of FFA is reduced by angiotensin (Mjos, Bugge Asperheim and Kiil, in press), and the same response will probably be found in other organs It therefore seems likely that changes in plasma FFA induced by angiotensin are due to reduced net release of FFA from adipose tissue

Angiotensin is a potent liberator of catecholamines from the adrenals (Feldberg and Lewis 1964, Robinson 1965, Peach *et al* 1966) and increases the amount of nor adrenaline released from sympathetic nerve terminals (Distler *et al* 1965) However, the pressor effect of angiotensin reduces sympathetic nerve activity In our study, the plasma FFA lowering effect of angiotensin was not further reduced after β adrenergic receptor blockade, indicating that in the intact dog the possible adrenergically mediated effect of angiotensin on lipolysis is of minor or no importance

Angiotensin was found to lower plasma FFA and to reduce FBF These effects were abolished by ganglionic blockade, when higher doses of angiotensin led to an increase in FBF and a relative rise in plasma FFA, accordingly, angiotensin caused parallel changes in blood flow and net release of FFA in adipose tissue Vasoconstriction induced by sympathetic nerve stimulation has been shown to reduce the rate of FFA release in subcutaneous adipose tissue, despite maintained or increased lipolysis (Rosell 1966) This is probably an effect of depressed transcapillary transport of FFA caused by reduced capillary surface area Similarly the plasma FFA lowering effect of angiotensin can be explained by its vasoconstrictive action Supporting this hypothesis is the overshoot occurring in plasma FFA after angiotensin infusion was discontinued which is compatible with a trapping of liberated FFA in adipose tissue *during* the infusion

In dogs pretreated with a ganglionic blocking agent, high doses of angiotensin induced an unexpected rise in FBF The underlying mechanism for this response is unclear, but the observation indicates that the vasoconstrictive effect of angiotensin in adipose tissue is less than in the body as a whole after ganglionic blockade

Atropine alone did not affect plasma FFA concentration, in contrast to Iizuka *et al*, the lowering effect of angiotensin on plasma FFA in our experiments was unaffected by atropinization This difference might be explained by the much lower initial values of plasma FFA in the atropinized group of Iizuka *et al* than in their untreated group, a slight (if any) fall in plasma FFA might therefore be expected in their atropinized group

From the experimental findings we conclude that the plasma FFA lowering action of angiotensin is secondary to its vasoconstrictive effect in adipose tissue

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We gratefully acknowledge the skilled technical assistance of Miss Jeanne Bonfield and Mr Bjørn Norstrand

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Specificity of Sensory Messages Mediated through Chorda Tympani Fibres with Multiple Sensitivity to Gustatory and Thermal Stimuli

By

H T Andersen and A O Hartmann

Received 4 March 1971

Abstract

Andersen, H T and A O Hartmann *Specificity of sensory messages mediated through chorda tympani fibres with multiple sensitivity to gustatory and thermal stimuli* Acta physiol scand 1971 *83* 150—155

Sensory messages about taste and temperature transmitted through single chorda tympani fibres have been studied by means of factor analysis using the varimax criterion for factor rotation Our analysis indicate that all sensory qualities studied *i e* warm cold salty sour, sweet and bitter are conveyed to the brain as separate sensory modalities No groups of interacting stimuli could be identified at the level of the primary afferent neuron It seems particularly important that the pattern of neural activity elicited by gustatory qualities may change over time so that the dynamic properties of the transmission system emerges as a possible mechanism of sensory discrimination Moreover this time dependent shift in the input of information to the central nervous system may give a clue to the phenomenon of aftertaste

Electrical activity recorded from the chorda tympani nerves of hamsters and rats shows that single afferent fibres may transmit sensory messages evoked by temperature as well as responses to one or more of the 4 gustatory primaries *i e* bitter, salty, sour and sweet (Ogawa Sato and Yamashita 1968) A search for interrelationships between these various sensory modalities was conducted by these authors The electrical activity recorded from each species was divided into 2 time periods The first time period encompassed the initial 5 sec after stimulation whereas the subsequent 5 sec interval *i e* 5—10 sec after a stimulus was applied to the tongue was included in the second period Each pair of stimuli was correlated across the single fibres from which registrations had been obtained The correlation matrixes of Ogawa *et al* (1968) thus correspond to the 4 primary gustatory qualities with temperature and spontaneous discharge added

However, the question of whether the nervous activity registered in gustatory afferents of hamsters and rats really fits into 4 independent sensory dimensions remains

TABLE I The factors in the data of Ogawa *et al* (1968)

No of Factor	Eigenvalue				% of variance				Cummulative percent			
	A	B	C	D	A	B	C	D	A	B	C	D
1	3 1	2 7	2 9	2 7	39	34	41	39	39	33	41	39
2	2 1	1 6	1 8	1 9	27	20	26	28	66	54	68	67
3	1 2	1 3	1 1	1 0	15	16	16	15	81	70	84	82
4	8	1 0	6	7	10	12	9	10	91	82	93	91
5	4	7	3	3	5	8	4	5	96	91	96	96
6	2	4	2	2	2	4	3	3	98	95	99	99
7	1	3	1	1	1	3	1	1	99	98	100	100
8	1	1	—	—	1	2	—	—	100	100	—	—

A Hamster, first time period
B Hamster, second time period
C Rat, first time period
D Rat, second time period

unsettled In order to investigate this problem further we have subjected the data of Ogawa *et al* (1968) to factor analysis using the varimax criterion for the factor rotations (Kaiser 1958, Harman 1967) The application of the method has been described in detail in a previous communication (Andersen and Hartmann 1971)

Results

The factorization of the correlation matrixes of Ogawa *et al*, (1968) is shown in Table I The cumulative percentages of the factors do not show evidence of any specific number of factors In accordance with the common concept of 4 gustatory modalites we started our analysis by rotating the 4 first factors

Table II and III show that the data do not constitute 4 monogustatory independent sensory modalities in our factor analysis Only salt and sweet tasting

TABLE II Rotation of 4 factors obtained from hamster

Factor No			1		2		3		4	
Data type			A	B	A	B	A	B	A	B
% of total variance			30	31	20	18	18	19	14	14
Variable	Communality		Factor loadings							
	A	B								
NaCl	95	84	−06	−17	−21	88	94	04	13	17
Sucrose	91	91	88	14	02	−46	−36	79	−08	−24
HCl	92	76	−13	−80	−90	33	26	−09	−14	03
Quinine	77	54	−53	−71	−68	15	15	−09	−06	−05
Saccharin	94	93	93	09	11	24	24	93	01	05
Cooling	91	81	19	−89	−91	−07	−07	−10	−22	−03
Warming	92	95	68	−01	−13	−13	−51	07	43	−96
Spontane	96	85	00	−71	−33	−51	−12	05	−92	30

TABLE III Rotation of 4 factors obtained from rat

Factor No			1		2		3		4	
Data type			C	D	C	D	C	D	C	D
% of total variance			27	35	26	27	15	15	24	14
Variable	Communality		Factor loadings							
	C	D								
NaCl	99	1 00	— 08	— 06	04	— 06	.99	1 00	— 03	03
Sucrose	96	97	04	— 01	—.95	97	— 23	14	05	00
HCl	87	78	48	85	— 04	04	06	— 02	— 80	23
Quinine	93	99	23	28	05	07	01	— 03	— 93	95
Saccharin	96	97	— 10	03	—.96	98	17	— 06	— 03	09
Cooling	89	84	90	91	09	— 05	— 09	08	— 25	05
Warming	—	—	—	—	—	—	—	—	—	—
Spontane	89	86	89	91	— 02	03	03	03	— 32	16

stimuli dominate separate factors This is true for both time periods in the hamster as well as in the rat When 5 factors were rotated the resolution was still poor However, with 6 factors, which account for 95 to 99 per cent of the total variance, a more distinct separation appears (Table IV and V)

Hamsters

The data obtained from the first time period after application of stimuli show that the various gustatory primaries dominate different factors (Table IV) Moreover, the activity elicited by warming and the spontaneous discharge appear separated Cooling loads primarily on the same factor as the sour stimulus in this initial time period, but in the second time period *i e* 5—10 sec after the various stimuli were applied, cooling shifts from the sour factor to that on which the spontaneous

TABLE IV Rotation of 6 factors obtained from hamster

Factor No			1		2	
Data type			A	B	A	B
% of total variance			23	21	23	17
Variable	Communality		Factor loadings			
	A	B				
NaCl	99	93	— 02	04	— 17	94
Sucrose	96	92	84	— 01	— 02	— 50
HCl	97	1 00	— 07	— 32	— 89	23
Quinine	1 00	99	— 31	— 19	— 39	04
Saccharin	97	94	94	09	04	23
Cooling	96	90	05	— 82	— 89	20
Warming	99	99	41	03	— 08	— 12
Spontane	1 00	93	01	— 92	— 28	— 20

discharge is preponderant This is the only noticable change in constituency observed to take place in the two time periods

Rats

In the rat a rather similar picture appears (Table V) All of the sensory modalities separate on distinctly different factors However, there is an obvious correlation between the response to cold stimulation and the spontaneous discharge since there is a definite tendency for them to load together The two time periods display a remarkable uniformity for the rat data

Rotation of more than 6 factors

Addition of the seventh factor was not found to resolve the different sets of data any further But in the hamster data which permit introduction of an eighth factor, we obtained a distinct separation of all stimuli used It should be emphasized however, that this observation pertain to the nervous activity recorded during the second time period only

Discussion

One conclusion appears inescapable from our analysis warming is transmitted as a sensory modality separated from all of the gustatory qualities Cooling also seems to be quite a specific modality although a certain correlation with sour exists The present lack of knowledge about receptor mechanisms makes further speculations about this possible interrelationship futile

The spontaneous discharge recorded by Ogawa *et al* (1968) may be a distinct property of certain single fibres, but in the present material it is obviously correlated with cooling This correlation could be artificially induced by low threshold cold receptors firing occasionally during the experiment Currents of air across the preparation might have induced such nervous activity later to be erroneously interpreted

3		4		5		6	
A	B	A	B	A	B	A	B
15	19	13	13	11	14	12	11
96	04	10	11	09	04	17	17
—37	78	—09	—21	—12	—09	—31	—09
20	—09	—09	03	31	29	16	87
12	—07	—09	04	84	95	15	22
19	94	04	05	—18	—03	—12	—04
03	—14	—31	—16	09	33	—24	27
—26	06	19	—98	—16	—03	—83	—07
—11	01	—94	15	07	03	13	13

TABLE V Rotation of 6 factors obtained from rat

Factor No			1		2	
Data type			C	D	C	D
% of total variance			17	18	26	27
Variable	Communality		Factor loadings			
	C	D				
NaCl	99	1 00	—06	—06	04	—06
Sucrose	96	97	03	—03	—95	97
HCl	1 00	1 00	30	35	—02	03
Quinine	1 00	1 00	20	14	03	07
Saccharin	96	97	—10	00	—96	98
Cooling	1 00	1 00	90	92	08	—03
Warming	—	—	—	—	—	—
Spontane	1 00	1 00	48	51	—01	03

as spontaneous discharge Moreover, the experimental procedure itself which involved application of water at 25° C to the tongue surface during determinations of the spontaneous discharge may be responsible for cold receptor activation particularly if evaporation occurred simultaneously

The phenomenon of after taste may be briefly commented upon using the results from our analysis of the hamster data over the two different time periods The resolution of data from the first time period *i e* the initial 5 sec after a stimulus was applied to the tongue did not yield more than 6 meaningful factors However, when the recordings obtained during the second time period 5—10 sec after stimulation, was analyzed 8 factors emerged Even the two sweet tasting stimuli sucrose and saccharine became separated The limitation in the data obviously makes it difficult to draw any strong conclusions from this observation It may be suggested however that the gustatory quality of saccharine changes with time at the level of the primary afferent in the hamster If this interpretation is correct it may be used to explain the distinct aftertaste which saccharine leaves The time dependent shift in gustatory quality which apparently takes place when saccharine is tasted support our previous suggestion that monogustatory stimuli may be difficult to define (Andersen and Hartmann 1971 Furthermore it has been previously emphasized that the nervous responses elicited by different taste stimuli may depend on their concentrations (Ganchrow and Erickson 1970 Andersen 1970) Thus the criteria for monogustatory properties of chemical compounds appear even further complicated

It should be noted also that when the transmission of gustatory messages changes over time already at the level of the primary afferent neuron this in itself may be a mechanism of discrimination between taste stimuli Undue emphasis on static gustatory neural patterns may cause a loss of the essential dynamic properties of taste specificity This becomes particularly important if the 'across fibre-pattern input into the primary gustatory afferents is used to explain the taste specificity mechanisms

[illegible]		4		5		6	
[illegible]	D	C	D	C	D	C	D
15	15	18	15	10	15	12	9
99	1 00	— 02	03	— 02	01	03	— 02
— 23	14	08	00	— 05	— 03	— 02	— 03
04	01	— 53	22	—.75	—.88	— 29	22
02	— 03	— 94	97	— 21	— 17	— 16	11
17	— 06	— 05	09	03	00	02	06
— 08	07	— 23	14	— 17	— 30	— 31	22
— 05	05	— 75	20	— 23	— 39	—.81	74

Finally, Ogawa *et al* (1968) *suggested that their findings might support the* duplexity theory of taste advanced by von Bekesy (1964) whose psychophysical experiments indicated that gustatory and thermal stimuli could be divided into two distinct groups When the proper stimuli were applied to the human tongue bitter, sweat and warm interacted among themselves to form one group whereas salty sour *and cold showed a similar interdependence Since* members of the one group never interacted with members of the other von Bekesy suggested that the different stimuli grouped together might have some quality in common *Our analysis do not indicate* any relationship between the neurophysiological data presented by Ogawa *et al* (1968) and the conclusions that von Bekesy (1964) arrived at on the basis of his psychophysical experiments even when possible species differences in taste discrimina tion are taken into account Nevertheless this apparent inconsistency between neuro physiological records obtained at *the level of the first order afferent* neuron and the results procured in psychophysical experiments may be fictitious Thus the central integration of sensory messages and the subsequent process of verbalizing the ex perience perceived by a human subject may cause the various independent sensory stimuli to cluster into groups of interacting modalities

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Comparison of the Effects of Prostaglandins E_1, E_2 and $F_{2\alpha}$ on the Sympathetically Stimulated Rabbit Heart

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Abstract

HEDQVIST, P and Å WENNMALM *Comparison of the effects of prostaglandins E_1 E_2 and $F_{2\alpha}$ on the sympathetically stimulated rabbit heart* Acta physiol scand 1971 *83* 156—162

PGE_1, PGE_2 and $PGF_{2\alpha}$ in concentrations ranging from 3×10^{-9} M to 1.5×10^{-6} M were infused into the isolated perfused, sympathetically innervated rabbit heart in order to assess their capacity to inhibit the chronotropic, inotropic and noradrenaline overflow responses to sympathetic nerve stimulation, as well as the chronotropic and inotropic responses to infusion of noradrenaline The coronary flow was increased by PGE_1 but not by PGE_2 of $PGF_{2\alpha}$ The three compounds did not change the heart rate or the contractile force, indicating that the reported increase in heart rate after i v infusion of PGE_1 or PGE_2 is reflex in origin. PGE_1 and PGE_2 inhibited the outflow of noradrenaline as well as the chronotropic and inotropic [illegible] None of [illegible]

the adrenergic nerve terminals

Prostaglandins E_1 and E_2 (PGE_1, PGE_2) have been shown to inhibit the mechanical as well as the noradrenaline (NA) overflow responses to sympathetic nerve stimulation in the cat spleen (Hedqvist and Brundin 1969, Hedqvist 1970 a) The two compounds depress the contractile response to transmural stimulation of the isolated guinea-pig and rabbit vas deferens (Euler and Hedqvist 1969, Hedqvist and Euler 1971), and the vascular response to activation of the vasoconstrictor fibres of the cat's hindleg (Hedqvist 1970 b) while not inhibiting the response to added NA. In preliminary communications the two compounds have been shown to inhibit the outflow of NA from the isolated rabbit heart in response to sympathetic nerve stimulation (Hedqvist Stjarne and Wennmalm 1970, Wennmalm and Hedqvist 1970)

The present paper reports in more detail the action of PGE_1 and PGE_2 on the innervated rabbit heart, and describes some quantitative differences in previously observed effects The actions of the two compounds was compared to that of $PGF_{2\alpha}$ in order to find out if inhibition of sympathetic neurotransmission can be achieved by other prostaglandins than those of the E type

Methods

99 rabbits of mixed strains and sexes, weighing from 1 3 to 2 3 kg, were used for the study

Atropine (10^{-6} g/ml) was administered at the beginning of the experiment The perfusion pressure was about 60 cm H_2O and the temperature 37° C The apex of the heart was connected to a strain gauge transducer and heart rate and myocardial contractile force were recorded on a Grass Model 5 D Polygraph The stimulation electrodes were platinum rings in the wall of a plastic tube, through which the tissue containing the sympathetic fibres to the heart was pulled In the tube, the tissue was continuously superfused by Tyrode's solution Electrical stimulation of the nerves was carried out with Grass Model S 5 stimulators, delivering 30 sec trains of rectangular pulses of 1 msec duration, at a frequency of 10/sec, and at supra maximal voltage The nerves were stimulated for two or three periods at 15 min intervals PGE_1 PGE_2 or $PGF_{2\alpha}$ in concentrations ranging from 3×10^{-9} M to 1.5×10^{-6} M were infused from 5 min before and to the end of the second stimulation The perfusate from the heart was collected during the stimulation periods and the following 90 sec. Representative samples were also taken from resting periods The collected perfusate was immediately chilled and acidified After purification on alumina the NA content was analyzed fluorimetrically (Euler and Lishajko 1961)

In other experiments

NA was expressed as the ratio between responses elicited during the second stimulation period (PG present), S_2 and the first stimulation period S_1 The S_2/S_1 ratios obtained were compared to S_2/S_1 ratios from control experiments, in which no infusion of PG was made during S_2

Results

Infusion of PGE_1 at a concentration of 3×10^{-7} M produced a moderate (49 ± 20 %, mean ± s e.m , n = 6), increase in the flow rate of the perfused rabbit heart Lower concentrations were ineffective The prostaglandins tested did not alter the frequency or contractile force of the spontaneously beating heart.

The effluent from the spontaneously beating heart did not contain NA in amounts detectable by the method used, neither in the absence nor in the presence of PGE_1, PGE_2 or $PGF_{2\alpha}$ at concentrations ranging from 3×10^{-9} M to $1\ 5\times10^{-6}$ M

During stimulation of the sympathetic nerves for 30 sec at a frequency of 10/sec considerable amounts of NA appeared in the effluent from the heart, usually about 150 ng As a result of the stimulation there was also a rapid and marked increase in heart rate and contractile force A second stimulation produced virtually the same chronotropic response, while the inotropic response and the NA overflow decreased somewhat. Infusion of PGE_1 or PGE_2 in a concentration of 3×10^{-9} M did not affect the response to sympathetic nerve stimulation However, at 3×10^{-8} M the two compounds inhibited the NA outflow in response to nerve stimulation and the inhibition progressively increased with the dose (Fig 1) Throughout the concentration range used, the inhibitory action of PGE_2 on the outflow of NA appeared less pronounced than that of PGE_1 However, the effects produced by equimolar concentrations of the two compounds were not significantly different The chronotropic response to nerve stimulation was depressed by PGE_1, 3×10^{-8} M, but not by PGE_2 at the same

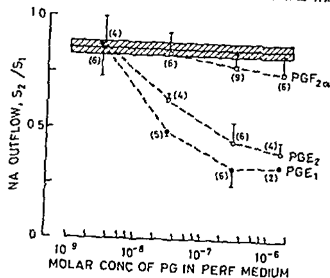


Fig 1 Perfused rabbit heart Effect of PGE_1, PGE_2 and $PGF_{2\alpha}$ on outflow of NA in response to sympathetic nerve stimulation (10/sec, 30 sec) All values presented as ratio between 2nd and 1st stimulation Hatched area Control expts in which PG's were omitted Vertical bars Means ± S E Figures within brackets Number of expts

concentration (Fig 2) In the higher dose range, 3×10^{-7} M to 1.5×10^{-6} M, PGE_1 and PGE_2 caused marked inhibition of the chronotropic and inotropic response (Fig 2, 3)

$PGF_{2\alpha}$ was infused into the heart in concentrations ranging from 3×10^{-8} M to 1.5×10^{-6} M In contrast to PGE_1 and PGF_2 this compound showed no effect on the outflow of NA in response to nerve stimulation, nor were the chronotropic or inotropic responses afffected (Fig 1, 2, 3)

In some experiments a third stimulation was given 15 min after the end of the infusion of prostaglandins By that time the inhibition produced by the PGE's was no longer apparent and the responses had returned towards the preinfusion levels (Fig 4)

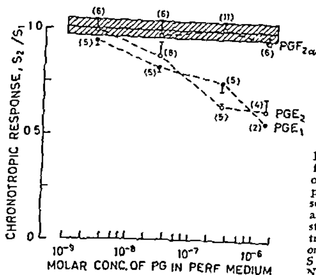


Fig 2 Perfused rabbit heart Effect of PGE_1, PGE_2 and $PGF_{2\alpha}$ on chronotropic response to sympathetic nerve stimulation (10/sec, 30 sec) All values presented as ratio between 2nd and 1st stimulation Hatched area Control expts in which PG's were omitted Vertical bars Means ± S E Figures within brackets Number of expts

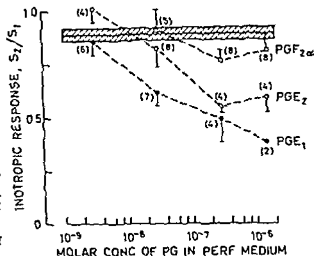


Fig 3 Perfused rabbit heart Effect of PGE_1, PGE_2 and $PGF_{2\alpha}$ on inotropic response to sympathetic nerve stimulation (10/sec, 30 sec) All values presented as ratio between 2nd and 1st stimulation Hatched area Control expts in which PG s were omitted Vertical bars Means±S.E Figures within brackets Number of expts

It thus seemed clear that PGE_1 and PGE_2 inhibit the effector responses to sympathetic nerve stimulation in the rabbit heart in part by a prejunctional action i e

... The total dose of NA administered was 0 45 μg or 2 7 μg, and it was infused in periods of 40 sec The lower dose increased mainly the contractile force while the higher dose increased both the heart rate and the contractile force PGE_1, 3×10^{-9} M to 3×10^{-7} M, and PGE_2, 3×10^{-9} M to $1\ 5\times10^{-6}$ M, were infused during the second NA infusion period Throughout the concentration range used the effects of PGE_1 and PGE_2 on the mechanical responses to NA were small and inconsistent, varying from slight inhibition to weak potentiation (Table I) $PGF_{2\alpha}$ ($4\ 8\times10^{-7}$ M) did not materially affect the mechanical response to NA

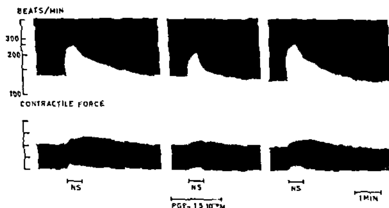


Fig 4 Perfused rabbit heart. Inotropic and chronotropic responses to three periods of nerve stimulation (10/sec, 30 sec) PGE_2 $1\ 5\times10^{-6}$ M infused during the second period

TABLE I Perfused rabbit heart Effect of PGE_1 and PGE_2 on chronotropic and inotropic responses to NA All values expressed as ratio between 2nd and 1st response (R_2/R_1), and given as means ± SE, n = number of observations

Stimulus NA µg/40 sec	Drug	Heart rate R_2/R_1	n	Contractile force R_2/R_1	n
0.45	—	—		0.92 ± 0.08	8
2.7	—	1.00 ± 0.07	7	0.88 ± 0.05	7
0.45	PGE_1 3×10^{-9}	—		0.79 ± 0.03	5
	3×10^{-8}	—		0.98 ± 0.12	6
	3×10^{-7}	—		0.95 ± 0.04	4
2.7	3×10^{-9}	1.06 ± 0.06	4	0.87 ± 0.05	5
	3×10^{-8}	1.05 ± 0.13	4	0.96 ± 0.09	6
	3×10^{-7}	0.91 ± 0.05	4	0.91 ± 0.06	4
0.45	PGE_2 3×10^{-9}	—		0.89 ± 0.10	4
	3×10^{-8}	—		0.84 ± 0.07	5
	3×10^{-7}	—		0.87 ± 0.08	4
	1.5×10^{-6}	—		0.91	2
2.7	3×10^{-9}	1.07 ± 0.11	4	0.99 ± 0.12	4
	3×10^{-8}	1.13 ± 0.08	4	0.85 ± 0.08	5
	3×10^{-7}	0.94 ± 0.03	8	0.89 ± 0.06	7
	1.5×10^{-6}	1.00	2	1.00	2

Discussion

The present study clearly demonstrates that PGE_1 and PGE_2 administered in low to moderate concentrations markedly depress the mechanical response to sympathetic nerve stimulation in the rabbit heart. This inhibitory action is in accordance with observations in several other sympathetically innervated tissues such as the rabbit oviduct (Brundin 1968), the cat spleen (Hedqvist and Brundin 1969, Hedqvist 1970 a) the vas deferens of the guinea-pig, the rabbit, the rat and the cat (Hedqvist and Euler 1971) and the vasculature of the cat's hindleg (Hedqvist 1970 b)

The inhibitory action of PGE_1 and PGE_2 appears to be mainly prejunctional and consist in reduction of the amount of transmitter secreted from the nerve terminals This conclusion is based on the following observations PGE_1 and PGE_2 reduced the outflow of NA in response to nerve stimulation at least to the same extent as they depressed the effector responses Moreover, the mechanical response induced by infusion of exogenous NA was not inhibited by PGE_1 and PGE_2 at the concentrations found to depress the response to nerve stimulation. Increasing doses of PGE_1 and PGE_2 caused a progressively more pronounced and parallel inhibition of the NA outflow and of the effector responses to nerve stimulation, and after the end of the infusion of PGE_1 and PGE_2 the NA outflow and the effector responses to nerve stimulation returned towards the preinfusion levels This is in agreement with observations where the time course for the PGE induced inhibition of NA release in response to sympathetic nerve stimulation is closely parallel to the inhibition of the effector responses, both with regard to onset and to recovery (Hedqvist and Brundin 1969, Hedqvist 1970 a)

$PGF_{2\alpha}$ administered at the same concentrations as PGE_1 and PGE_2 did not affect the outflow of NA in response to nerve stimulation, nor did it alter the mechanical responses induced by nerve stimulation or by infusion of NA Similar observations have been made in the guinea pig vas deferens where PGE_1 and PGE_2 are at least 1000 times more active than $PGF_{2\alpha}$ as inhibitors of the neuromuscular transmission (Hedqvist and Euler 1971)

PGE_1 has been reported to increase the coronary flow in the guinea pig the rat and the cat (Berti, Lentati and Usardi 1965, Vergroesen, De Boer and Gottenbos 1967, Hollenberg, Walker and McCormick 1968, Nakano 1968) In the present experiments with the rabbit heart PGE_1 but not PGE_2 or $PGF_{2\alpha}$ increased the flow rate The difference in effect between PGE_1 on the one hand and PGE_2 and $PGF_{2\alpha}$ on the other is in accordance with previously reported differences in their vasoactivity in general (*cf* Bergstrom, Carlson and Weeks 1968)

In the present study PGE_1, PGE_2 and $_2FGP_\alpha$ did not alter the heart rate which justifies a comment on the cardiovascular effect of the compounds in the rabbit Intravenous infusion of PGE_1 and PGE_2 into the rabbit produces a depression of the systemic arterial blood pressure and simultaneously an increase in the heart frequency (Bergstrom and Euler 1963) In view of the present results it seems clear that the latter effect cannot be explained in terms of a direct action on the heart and thus must be considered as reflex in origin and secondary to the fall in blood pressure

The present results support previous findings that exogenous PGE_1 and PGE_2 markedly and reversibly inhibit the sympathetic neuromuscular transmission in many tissues, mainly by a prejunctional action (for references see under introduction) and thus increase the probability for the hypothesis that endogenous PGE_1 and PGE_2 may act as physiological modulators of sympathetic neurotransmission (Hedqvist and Brundin 1969, Hedqvist 1970 a) Several lines of indirect evidence are compatible with such a role for endogenous prostaglandins (Hedqvist 1970 c, Hedqvist and Euler 1971, Wennmalm 1971, Wennmalm and Stjarne 1971) However final experimental proof that such a control mechanism exists and operates at a physiologically relevant level is still lacking

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Increased Nerve Stimulation Induced Release of Noradrenaline from the Rabbit Heart after Inhibition of Prostaglandin Synthesis

By

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Abstract

Samuelsson, B and A. Wennmalm *Increased nerve stimulation induced release of noradrenaline from the rabbit heart after inhibition of prostaglandin synthesis* Acta physiol scand 1971 *83* 163—168

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Prostaglandins of the E series (PGE_1, PGE_2) have been shown to inhibit the release of noradrenaline (NA) in response to nerve stimulation in various tissues and species Thus in the isolated cat spleen, PGE_1 and PGE_2 depress the nerve stimulation induced release of adrenergic transmitter (Hedqvist and Brundin 1969, Hedqvist 1970 a) and the same inhibitory action has been found in the isolated perfused rabbit heart (Wennmalm and Hedqvist 1970, Hedqvist, Stjarne and Wennmalm 1970) In addition, PGE_1 and PGE_2 depress the vascular response to sympathetic nerve stimulation in the blood perfused cat hind leg (Hedqvist 1970 b) and the smooth muscle response to transmural stimulation of the isolated guinea pig vas deferens preparation (Euler and Hedqvist 1969) Recently it has been shown (Wennmalm and Stjarne 1971) that sympathetic nerve stimulation causes an out-

flow of prostaglandin like material from the isolated perfused rabbit heart in con centrations sufficient to inhibit the nerve stimulation induced outflow of NA from a second heart From these observations it has been suggested (Hedqvist and Brundin 1969, Hedqvist 1970 c, Wennmalm and Stjarne 1971) that the release of adrenergic transmitter on depolarization is normally limited by an endogenous, prostaglandin mediated mechanism

Conclusive evidence for the existence of such a mechanism requires the use of specific inhibitors of the synthesis of prostaglandins The present paper reports the outflow of NA released by nerve stimulation when 5,8,11,14 eicosatetraynoic acid (ETA), a compound known to block prostaglandin synthesis *in vitro*, (Downing Ahern and Bachta 1970, Hamberg and Samuelsson, unpubl obs) was used to inhibit *prostaglandin synthesis*

Methods

35 rabbits of mixed strains and sexes were used for the study They were killed by a blow on the head and bled from the left carotid artery The heart with intact left and right sympathetic nerve supply was dissected out according to Loffelholz and Muscholl (1969) The organ was perfused at a temperature of 37° C and a pressure of about 60 cm H_2O with Tyrode s solution (conc in mM NaCl 136 9, KCl 2 7, $CaCl_2$ 1 8, $MgCl_2$ 1 0, $NaHCO_3$ 11 9 NaH_2PO_4 0 4 glucose 5 6) aerated with 6 5 % CO_2 in O_2 Ascorbic acid 20 μg/ml was added to the solution The nerves with adjacent tissue were pulled through separate plastic tubes with platinum rings in their walls serving as electrodes and connected to Grass Model S5 stimulators They were stimulated by rectangular pulse trains of supramaximal strength and 1 msec duration. The apex of the heart was connected to a strain gauge transducer and heart rate and contractile force were recorded on a Grass Model 5D Polygraph. The effluent from the heart was collected during the nerve stimulations and until the contractile response had faded out. ETA in ammonium form (0 2—0 6 mg/ml), was infused through a cannula immediately above the aorta to produce final concentrations of 8×10^{-6} M to 10^{-8} M

12 hearts were used for studying the influence of ETA on the outflow of NA in response to nerve stimulation They were stimulated by 5 stimuli/sec for 6 min The effluent from the heart was collected in 2 min periods and immediately acidified and chilled In 6 hearts ETA was infused from the beginning of the second 2 min effluent collection period and for the following 3 min. The effluents were purified on alumina and assayed for NA according to Euler and Lishajko (1961) The ratio outflow of NA during the second or third 2 min [illegible] infused with [illegible] rd period [illegible] ts t test. [illegible] lins from the organ on nerve stimulation 11 hearts served as donors of prostaglandin which was extracted and tested for PG activity on 6 hearts The donor hearts were initially infused with ETA for 2 to 5 min After about 5 min of washing they were stimulated by 5 stimuli/sec for 3—5 min with 15 min intervals The effluent from the heart during the stimulation periods [illegible] with equal volumes [illegible] fer (pH 6 5) and [illegible] to dryness The [illegible] prepared from the [illegible] ve stimulation in [illegible] in resting period [illegible] was collected from [illegible] out It was in- [illegible] extract described above was infused and a *second nerve stimulation was performed during infusion* of the lipid extract 15 min after the first stimulation The effluent was collected as earlier The ratio outflow of NA during the second stimulation/outflow of NA during the first stimulation was used as a standard of the capacity of the extract to inhibit the outflow of NA in response to nerve stimulation from the heart The ratio was compared to the corresponding value obtained

in an earlier experimental series where ETA was not infused. The difference between the ratios obtained when ETA was used and the controls was statistically analyzed using Student's t test.

3 hearts were used to test if ETA affects the uptake of exogenous NA in the organ. After a 15 min resting period they were perfused for 10 min with a Tyrode's solution containing 5 µC l-NA-^{3}H (Amersham) and 25 µg NA per liter. After this, perfusion was continued with NA free Tyrode's solution, and ETA (5×10^{-6} M to 10^{-5} M) was infused for 12 min. After 2 min of ETA infusion, perfusion was switched to NA-containing Tyrode's solution. During the two periods of perfusion with labelled and unlabelled NA, 0.5 ml aliquots of the effluent were taken after 4, 7 and 10 min. They were added to a 7 : 3 toluene-absolute ethanol solution containing 4 g of 2-5-diphenyloxazole and 100 mg 1-4 bis-2(4 methyl-5-phenyloxazolyl)benzene per liter of toluene. The samples were counted in a Packard Tri Carb Liquid Scintillation Spectrometer.

In 1 expt., perfusate from 3 stimulated hearts not previously infused with ETA was collected. Extraction of lipids was performed as mentioned above and the extract was purified using [illegible] on on thin layer [illegible] and PGE_2 areas [illegible] me, diluted with [illegible] xtracted 3 times [illegible] to dryness. The [illegible] was divided in 4 fractions and one of them was incubated at 37° C for 2 hrs with 15-hydroxyprostaglandin dehydrogenase and NAD^+. The remaining 3 fractions served as controls and were incubated similarly, one of the fractions without enzymes, and the others with 15 hydroxyprostaglandin dehydrogenase or NAD^+ only (Änggård and Jonsson 1971).

Results

Stimulation of the sympathetic nerves to the heart for 6 min at a frequency of 5 stimuli/sec caused a marked increase in heart rate and contractile force, and in addition an outflow of NA, 116 ± 31 ng (mean ± SE, n = 10) during the first 2 min period of stimulation. During the following two 2 min periods the outflow of NA declined somewhat, to 80 ± 5 % (mean ± SE, n = 5) during the second and to 53 ± 7 % (n = 4) during the third 2 min period, of the outflow during the first 2 min period. When ETA was infused during 3 min from the beginning of the second period the outflow of NA induced by nerve stimulation increased to 135 ± 7 % (n = 5) during the second and 119 ± 11 % (n = 4) during the third 2 min period relative to the first stimulation period (Table I). The difference in outflow of NA during the second and third period, relative to the first, between ETA and control experiments is statistically significant (P = 6.67***, n = 10 and P = 5.26**, n = 8 respectively).

TABLE I. [illegible]

	0—2 min	2—4 min	4—6 min
Controls	100	80 ± 5 (5)	53 ± 7 (4)
ETA infused during min 2—5	100	135 ± 7 (5)	119 ± 11 (4)

TABLE II Perfused rabbit heart Ratio between outflow of NA during 2nd and 1st nerve stimulation (300 stimuli at 10/sec) in control experiments in experiments where the 2nd nerve stimulation was performed during infusion of acid lipid extract of stimulation perfusate (SE) and in experiments where the 2nd nerve stimulation was performed during infusion of acid lipid extract of stimulation perfusate from hearts previously treated with ETA Values given as mean ± SE Figures within brackets number of expts

	Controls	SE	ETA SE
S_2/S_1	0 88 ± 0 03 (8)	0 63 ± 0 06 (6)	1 05 ± 0 10 (6)

Infusion of lipid extracts of perfusate from ETA treated donor hearts caused no change in outflow of NA during the second nerve stimulation (ratio between outflow during second and first stimulation 1 05 ± 0 10, mean ± SE, n = 6), as compared to results obtained in a previous series (Wennmalm 1971), where no extract was infused prior to the second stimulation (ratio between outflow during second and first stimulation 0 88 ± 0 03, n = 8) However, infusion of lipid extracts of perfusate from sympathetically stimulated hearts not exposed to ETA causes a decrease in the nerve stimulation induced outflow of NA (Wennmalm and Stjärne 1971) In those experiments the ratio between the outflow figures during the second (extract infused) and first stimulation was 0 63 ± 0 06 (n = 6) The difference between this figure and that obtained in the present ETA experiments is statistically significant (P = 3 62**, n = 12) (Table II)

In control experiments 89 ± 4 % (mean ± SE) of exogenous NA infused appeared in the effluent from the heart When ETA was infused from 2 min before and during the second NA infusion 82 ± 6 % of the amine appeared in the effluent Thus, there was no evidence that ETA blocks the uptake of exogenous NA

The 4 samples incubated at 37° C for two hrs were tested for biological activity on an isolated rat stomach No smooth muscle stimulating activity was found in the fraction incubated with both 15 hydroxyprostaglandin dehydrogenase and NAD The 3 control fractions were all found biologically active Since 15 hydroxyprostaglandin dehydrogenase is specific for the 15 hydroxyl group in the compound and since the resulting product 15 keto-PG is biologically inactive the result obtained demonstrates the presence of PG in the effluent from sympathetically stimulated hearts

The extracts from thin layer chromatography were similarly tested for smooth muscle stimulating activity Both extracts were found biologically active indicating the presence of both PGE_1 and PGE_2 in the perfusate from sympathetically stimulated hearts

Discussion

It is well established that prostaglandins of the E series inhibit the nerve stimulation induced outflow of NA from the rabbit heart (Wennmalm and Hedqvist 1970 Hedqvist and Wennmalm 1971) By analogy with the action of PGE_2 in the cat spleen

(Hedqvist 1970 a) it seems probable that this inhibitory action is due to a depressed release of NA from the adrenergic neurons

Stimulation of the splenic nerves to the isolated dog or cat spleen has been shown to cause a release of PGE_2 from the organ (Davies, Horton and Withrington 1968, Gilmore Vane and Wyllie 1968) Similar results have been obtained in the isolated perfused rabbit heart (Wennmalm and Stjarne 1971) where stimulation of the sympathetic nerves caused an outflow of prostaglandin like material from the organ in amounts sufficient to depress the nerve stimulation induced outflow of NA from a recipient heart perfused with the effluent from the donor heart The active principle has now been shown to be both PGE_1 and PGE_2

In the present investigation the endogenous synthesis of prostaglandins was inhibited by administering ETA which has been shown to block synthesis of prostaglandins *in vitro* The failure of lipid extracts of perfusate from stimulated ETA-treated hearts to depress the release of NA in response to nerve stimulation indicates that prostaglandin synthesis was efficiently blocked

When ETA was infused during continuous sympathetic nerve stimulation of the heart, the outflow of NA from the organ was enhanced This cannot possibly be explained in terms of blocked reuptake of transmitter, since ETA did not depress the uptake of exogenous NA Neither can the increased outflow of NA caused by ETA be due to diminished enzymatic inactivation of the transmitter, since in this preparation the NA released in response to nerve stimulation is enzymatically degraded to less than 10 % (Wennmalm and Stjarne 1971) Thus it seems most likely that ETA treatment augments the liberation of transmitter in response to depolarization

The finding that inhibition of the local formation of prostaglandins is accompanied by an augmented secretion of transmitter per nerve impulse seems to provide conclusive evidence for the existence of a prostaglandin mediated control mechanism normally operating on the neurons to limit transmitter release

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The Effect of Glucagon on Intestinal Motility and Blood Flow

By

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Abstract

FASTH, S and L HULTEN *The effect of glucagon on intestinal motility and blood flow* Acta physiol scand 1971 *83* 169—173

[illegible] mooth muscle tone was [illegible] volume recording device [illegible] a a drop recording unit [illegible] A femoral artery was [illegible] close intraarterially at [illegible] act glucagoninfusion was followed [illegible] od flow and a somewhat delayed [illegible] renals were excluded glucagon still [illegible] the blood pressure increase were both abolished however It is therefore suggested that glucagon exerts its action on the splanchnic area in two different ways on the one hand by a direct effect on the vascular smooth muscles on the other hand by a release of catecholamines from the adrenal medullae which, in turn inhibits intestinal smooth muscles

In recent years considerable interest has been focussed on the functional significance of glucagon, an essential hormone (Sokal 1966) produced in the pancreatic α-cells Beside its wellknown effects on carbohydrate metabolism glucagon also appears involved in the regulation of gastrointestinal secretion, motility and blood flow Thus it inhibits pancreatic secretion (Dyck *et al* 1969) and also gastric acid secretion both in animals and man (Clarke, Neill and Welbourn 1960, Solomon and Spiro 1959), while pepsin output seems to be unaffected in man (Brooks Isenberg and Grossman 1969)

In most species including man, administration of glucagon inhibits motility in the stomach the small intestine and the colon (Stunkard van Itallie and Reis 1955 Dotevall and Kock 1963, Necheles Sporn and Walker 1966) It further seems to induce an intense increase of blood flow predominantly in the splanchnic vascular bed (Kock, Tibblin and Schenk, Jr 1970)

Whereas the hemodynamic effects of glucagon on the splanchnic vascular bed have been extensively studied previously (Tibblin, Kock and Schenk, Jr 1970) the mechanism by which glucagon inhibits instestinal smooth muscle tone has not been specifically dealt with so far Stunkard, van Itallie and Reis (1955), who

studied its inhibitory effects on gastric hunger contraction in man suggested that this effect could not be ascribed to the hyperglycaemia and concluded that it was either the result of a direct effect on the smooth muscles or indirectly related to the peripheral glucose utilisation Sarcioni *et al*, (1963) observed, however, that glucagon administration was regularly followed by a significant rise of circulating catecholamines, known to effectively inhibit gastrointestinal motility (Kock 1959) Recently, the adrenal release of catecholamines as induced by glucagon has in fact been recommended as a clinical aid in the diagnosis of pheochromocytoma (Lawrence 1967)

The aim of the present investigation was to study in more detail the qualitative and quantitative effects of graded glucagon infusions on intestinal motility and blood flow and to provide evidence for the hypothesis that adrenal catecholamine secretion might mediate the intestinal inhibition caused by glucagon administration

Methods

Experiments were carried out on 23 anesthetized cats (chloralose 50 mg/kg) fasted for 24 hrs A jejunal segment
exstirpated together w
and a femoral artery
pressure In one series
(11 cats) the splanch
cholamine secretion from the adrenal medullae was completely eliminated by isolating both adrenals from the circulation by encircling ligatures

Recording of motility Intestinal motility was recorded by a volume recording device at a constant intraluminal pressure at 10—12 cm H_2O The pressure reservoir was connected to a pistonrecorder writing on a kymograph

Recording of blood flow To measure quantitatively overall intestinal blood flow the left carotid artery was connected via a drop recording unit with the superior mesenteric artery By using siliconized tubes of wide dimensions and short length the resistance of this device for recording arterial inflow was kept minimal

Administration of drugs In all experiments glucagon was administered as close intraarterial infusions at different rates To abolish the sympathetic nervous influence on the vascular bed guanethidine (Ismelin® Ciba) was in some experiments administered i v in a dose of 3—5 mg/kg b w known to eliminate the release of catecholamines from the vasoconstrictor nerve endings

Result

A Adrenals intact As is shown in Fig 1 intraarterial infusion of glucagon induced a considerable and immediate increase of intestinal blood flow With about one minute delay a pronounced inhibition of motility occurred, concomitantly with a small but significant rise of arterial blood pressure This characteristically delayed response occurred regularly using doses between 10 and 100 µg/kg/min, but it tended to subside somewhat upon repeated infusions of glucagon

B Adrenals denervated or exstirpated As is shown in Fig 2 (upper panel) the intestinal blood flow increase and the delayed motility inhibition and blood pressure increase as a result of glucagon infusion occurred to the same extent also if the adrenal glands were denervated by severing all the splanchnic nerves

Exclusion of the adrenals by encircling ligatures eliminated the delayed motility

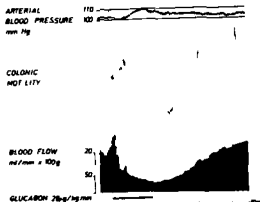


Fig 1 Cat 2 4 kg Adrenals intact Effects of i a glucagon infusion on simultaneous recording of systemic arterial blood pressure colonic motility and blood flow Note the slight increase in arterial pressure, the somewhat delayed but profound inhibition of motility and the immediate increase of blood flow

inhibition and blood pressure rise while the intestinal blood flow increase upon glucagon infusion remained virtually the same (Fig 2, lower panel) After guanethidine in doses that entirely bloc adrenergic transmission glucagon still induced a significant blood flow increase.

Discussion

In man as well as in animals glucagon has previously been shown to inhibit gastric, small intestinal and colonic motility, (Sporn and Necheles 1956 Kock, Darle and Dotevall 1967) In these studies the mechanism by means of which glucagon exercises its inhibitory influence on intestinal motility could not be clearly settled, however It was concluded that the inhibitory effect could not possibly be ascribed to the concomitantly induced hyperglycaemia In rat experiments, Lefebvre and Dresse (1961) showed evidence that glucagon had an action on the adrenal glands insofar as glucagon injections lead to a reduced amount of catecholamines in the adrenal medullae It was also observed that adrenalectomy abolished the antiphlogistic effects of glucagon in rat Lefebvre (1961), indicating that this effect was also mediated via the adrenal glands Furthermore the inhibitory effect of glucagon on the spontaneous contractions of the rat uterus was absent after adrenalectomy (Dresse and Lefebvre 1961)

In view of these facts it seems reasonable that the intestinal inhibition induced by glucagon might also be mediated via a release of catecholamines from the adrenal medulla The present investigation strongly suggests that this is so since the intestinal inhibition was abolished by adrenalectomy According to Kock *et al* (1967) the intestinal inhibition in man starts some 30—60 sec after i v glucagon injections In the present experiments where glucagon was given intraarterially to the intestine the latency of intestinal inhibition was still longer (60—90 sec), which is in accordance with the expected delay for a mediation via catecholamine release from the adrenal glands

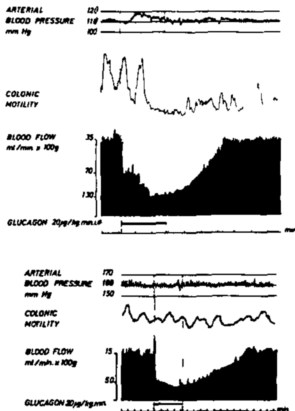


Fig 2 Effects of i a glucagon infusion on simultaneous recording of systemic arterial blood pressure, colonic motility and blood flow Upper panel Cat 3 4 kg Splanchnic nerves cut but adrenals intact Note the slight increase in arterial pressure the somewhat delayed but profound inhibition of motility and the immediate increase in blood flow

Lower panel Cat 3 4 kg Adrenals exstirpated Note the absence of motility and blood pressure changes in contrast to the marked increase in blood flow

In an extensive study on mechanisms engaged in reflex inhibition of intestinal motility Kock (1959), suggested that such intestinal inhibition is to a great extent due to the release of catecholamines from the adrenal medullae, particularly as concerns the direct adrenergic effect on the smooth muscles In the present study the intestinal inhibition following intraarterial glucagon infusion differed from the above mentioned reflex inhibition in one important respect, however Whereas intestinal reflex inhibition is dependent on intact adrenal sympathetic innervation severing all sympathetic nerves to the intestine and those to the adrenal glands did not interfere with the inhibitory response obtained by glucagoninfusion This indicates that the catecholamine release induced by glucagoninfusion is probably obtained by a direct stimulating effect on the adrenal medullae

It has previously been reported that repeated injections of glucagon diminish or even eliminate the intestinal inhibitory responses (Stunkard *et al* 1955 Dresse and Lefebvre 1961) also observed in the present study The mechanism for this is not clear, but it by no means interferes with the evidence that the inhibitory effect is conveyed by means of catecholamine release

Furthermore, as long as the adrenals were intact glucagon injection resulted in a slight but significant increase in blood pressure, an effect that was abolished

by adrenalectomy This indicates that the positive inotropic and chronotropic effect of glucagon reported by Farah and Tuttle (1960) might also to a great extent be secondary to a catecholamine release from the adrenal medullae and not only an effect of locally released myocardial epinephrine as was suggested by these authors In contrast to the delayed intestinal inhibition and increase in blood pressure, the increase of intestinal blood flow occurred almost immediately and was quite unaffected by either adrenalectomy or by repeated glucagon injection Furthermore, guanethidine administered in a dose known to abolish all sympathetic nervous influence on the vascular bed, did not significantly affect the increase of intestinal blood flow after glucagon infusion

Thus, glucagon seems to exert its action on the splanchnic area in two different ways on the one hand, by a direct effect on the vascular smooth muscles on the other hand by a release of catecholamines from the adrenal medullae which, in turn inhibits the activity of the instestinal smooth muscles

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Salivary Secretion in the Rat in a Hot Environment

By

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Abstract

ELMÉR, M and P OHLIN *Salivary secretion in the rat in a hot environment* Acta physiol scand 1971 *83* 174—178

Conscious rats were found to secrete saliva from the submaxillary gland when exposed to heat. In rats with sympathetically denervated glands the secretory rate was a little lower and in parasympathetically denervated glands the secretion was almost abolished The salivary flow appeared to increase with increasing ambient temperature and the secretion started earlier at higher temperatures The secretion elicited by heat stress was compared to the secretion evoked by electrical stimulation of the parasympathetic nerve to the gland It is concluded that the salivary secretion evoked by heat stress is mainly mediated by the parasympathetic secretory nerves, although the sympathetic fibres also take part

Rats do not sweat or pant in a hot environment, but increase heat loss by spreading saliva onto their body surfaces (Hainsworth 1967 and 1968, Hainsworth *et al* 1968) Evaporation of salivary water is elevated in the heat This is the case even if the parotid ducts are ligated, but not when the submaxillary sublingual glands are surgically removed (Hainsworth and Stricker 1969) In rats where the submaxillary sublingual glands are removed and in rats with bilateral destruction of the chorda tympani the heat resistance is markedly lowered (Stricker and Hainsworth 1970) These findings indicate a salivary secretion in the heat, especially from the submaxillary-sublingual glands, mediated at least in part by the parasympathetic secretory fibres Reflex secretion in rats may be elicited not only via the parasympathetic but also via the sympathetic glandular nerves (Ohlin 1968)

The normally innervated submaxillary gland shows a marked hypertrophy in rats exposed to heat for three weeks while the increase in weight in parasympathetically denervated glands is much less In the sublingual gland no corresponding hypertrophy is observed, which may indicate that this gland does not take part in the body temperature regulation by saliva spreading in the heat It seems likely that the hypertrophy of the submaxillary gland is to a large extent secondary to an increased secretory activity elicited by the parasympathetic secretory fibres (Elmér and Ohlin 1970)

Salivary secretion in the rat in a hot environment has thus far only been estimated by studying the evaporative water loss from the whole animal In the present investigation the secretory activity of the submaxillary gland evoked by heat stress was directly measured in conscious rats and in order to evaluate the role of the different autonomic glandular nerves the glands were parasympathetically or sympathetically denervated or both

Methods

27 female albino rats of the Sprague Dawley strain were used The animals, weighing about [illegible]

parasympathetic denervation was made by section of the chorda lingual nerve 4 rats were both sympathetically and parasympathetically denervated 7 normally innervated rats served as controls

About one hr after the operation when the rats had recovered from the anesthesia they were placed in a room with a constant temperature of 34° C 36° C 40° C or 44° C Each animal was kept in a metal cage and drops of saliva appearing at the tip of the cannula were marked on a smoked drum

In 3 rats the secretory response of the submaxillary gland to electrical stimulation of the chorda lingual nerve was studied in chloralose anesthesia A Grass stimulator, giving rectangular pulses of supramaximal intensity 2 5—5 0 V, with a duration of 1 5 msec and with a frequency of 1—30 shocks/sec was used

Results

When the rats were exposed to an ambient temperature of 40° C the secretion from the submaxillary gland started after 35—45 min and ceased after 75—85 min The animals died after 90—100 min exposure to 40° C (Table I) No significant difference was found between the rats with denervated glands and those with normally innervated glands regarding the time of beginning and end of secretion and survival time

The maximal secretory rate of the normally innervated gland in 40° C was found to be about 3 5 drops of saliva per minute (Table I) Sympathetic denervation reduced the maximal secretory rate significantly to about 63 % of controls and parasympathetic denervation to about 3 % (Fig 1) Both sympathetic and parasympathetic denervation of the same gland caused approximately the same reduction of the secretion as parasympathetic denervation alone The salivary flow usually reached its maximum within 10 min after the secretion had started then showed large variations and finally stopped about 15 min before the death of the animal

3 rats were exposed to an ambient temperature of 36° C In one with normally innervated submaxillary glands the secretion started after 95 min and the maximal secretory rate was 2 4 drops/min Two rats with sympathetically denervated glands started secretion after 85 and 90 min respectively and both reached a maximal

TABLE I Secretory activity of normally innervated, sympathetically parasympathetically and totally denervated submaxillary glands in rats exposed to an ambient temperature of 40° C Values are mean ± standard error of mean

Denervation	Number of rats	Time of start (min after *entrance to* hot environment)	Maximal secretory *rate* (drops/min)	End of secretion (min)	Time of death (min)
Controls (Normally innervated)	4	35 ± 6 5	3 5 ± 0 56	74 ± 9 0	89 ± 5 5
Sympathetic	7	36 ± 4 4	2 2 ± 0 20[1]	86 ± 8 9	99 ± 7 8
Parasympathetic	4	45 ± 5 4	0 1 ± 0 05[2]	77 ± 7 9	85 ± 4 4
Sympathetic + Parasympathetic	4	36 ± 2 5	0 2 ± 0 06[2]	74 ± 2 4	87 ± 5 9

[1] $p < 0\ 05$
[2] $p < 0\ 001$ when compared to controls

secretory rate of 2 drops/min They were still alive and secreting saliva after 300 min exposure to 36° C

In order to study the secretion at the temperature used in the hypertrophy experiments of Elmer and Ohlin (1970) one normally innervated rat was tested at 34° C The secretion started after 180 min and the maximal secretory rate was 1 drop/min. After 320 min the animal was still alive and secreting saliva

In one normally innervated rat exposed to 44° C the salivary secretion started after 10 min stopped after 35 min and the animal died after 40 min The maximal secretory rate was 4 5 drops/min

Electrical stimulation of the chorda lingual nerve caused a secretion of saliva from the submaxillary gland as described earlier (Ohlin 1965) The maximal secretory rate was 3 8 ± 0 44 drops/min reached at a frequency of 20 shocks/sec The secretory rate produced by exposing the sympathetically denervated rats to 40 C, 2 2 drops/min corresponds to an impulse frequency in the chorda lingual nerve of about 10 shocks/sec The secretion in sympathetically denervated rats in 36° C 2 drops/min corresponds to about 5 shocks/sec in the chorda lingual nerve

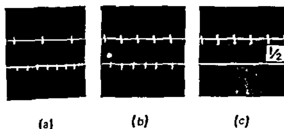


Fig 1 Secretion from the submaxillary gland of rats exposed to an ambient temperature of 40° C (a) Normally innervated (b) sympathetically denervated (c) parasympathetically denervated Records from above time in min drops of saliva

Discussion

Use of saliva for cooling is of vital importance for the control of body temperature in the Virginia opossum (Higginbotham and Koon 1955) and the rat (Hainsworth 1967) Direct measurements of salivary secretion in the heat have hitherto not been made in rodents, but increased salivary flow in response to heat stress has been directly demonstrated in the dog (Gregersen 1931) and buffalo (Aliev 1960)

The beginning of the secretion of saliva from the submaxillary gland of the rat after about 35—45 min in 40° C is in accordance with the finding that evaporative water loss from rats with ligated parotid ducts begins to increase after 40 min in the same ambient temperature (Hainsworth and Stricker 1969) Normal rats survive for 4—6 hrs in 40° C Some rats, however, which do not groom in the heat but swallow the saliva or let it flow down their chests only survive for about 120 min Submaxillary sublingual desalivated rats are unable to withstand exposure to 40° C for more than 45—90 min (Hainsworth *et al* 1968) It is the survival times of these rats, lacking saliva spreading behaviour that should be compared to the rats of the present investigation, because the latter were immobilized in their cages and prevented from spreading saliva on their fur and skin They died after 90—100 min, *i e* later than desalivated rats but somewhat earlier than the rats lacking the saliva spreading behaviour

Reflex submaxillary secretion in rats can be evoked both via the parasympathetic and sympathetic glandular nerves (Ohlin 1968) Parasympathetic denervation caused a marked reduction of the salivary secretion elicited by heat stress, indicating that the parasympathetic nerves are responsible for the main part of the effect Both sympathetic and parasympathetic denervation of the same gland caused approximately the same reduction of the secretion Sympathetic denervation, however, also reduced the secretory response to heat significantly This is in consistency with the finding that the hypertrophy of the submaxillary gland in rats exposed to heat for a long time is much less in parasympathetically denervated glands than in normal ones and that completely denervated glands show no hypertrophy at all (Elmer and Ohlin 1970)

The small secretion seen at 40° C after complete denervation of the glands may have been caused by circulating catecholamines released from the adrenal medulla in response to the heat stress Intravenously administered adrenaline elicits submaxillary secretion in the rat (Ohlin 1965) In the experiments of Elmer and Ohlin (1970), no hypertrophy was found in completely denervated glands at 34° C At this low temperature probably not enough catecholamines were released to induce salivary secretion

Salivary output inferred from measurements of evaporative water loss increases with increasing ambient temperature (Hainsworth 1968) This observation was supported by the present investigation At 36° C 40° C and 44° C the maximal secretory rates were 2 4 drops/min 3 5 drops/min and 4 5 drops/min respectively and the secretion started earlier at higher ambient temperatures

The calculated salivary flow rate at 40° C is about 35 μl/min or 0 27 μl per mg

gland per min in a normally innervated submaxillary gland, approximating the flo from a single submaxillary-sublingual gland calculated from evaporative water lo (Hainsworth and Stricker 1969) The wet weight of a submaxillary gland is abo 130 mg in a 200 g rat (Elmér and Ohlin 1969) In sympathetically denervated rat exposed to 40° C the flow rate was 0 17 μl per mg per min This flow corresponds t an electrical stimulation of the parasympathetic nerve to the gland with an impul frequency of 10 shocks/sec The maximal secretory rate 0 29 μl per mg per mi found at an impulse frequency of 20 shocks/sec is in accordance with similar expen ments using a glass cannula in the submaxillary duct of rats in chloralose anesthesi (Ohlin 1965)

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Unidirectional Fluxes in Isolated Splenic Nerve Vesicles Measured by a Millipore Filter Technique:

Effects of noradrenaline and competitive reversal of reserpine inhibition

By

Richard L Klein and Hugo Lagercrantz

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Abstract

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Isotopically labelled [illegible]

kinetics are compatible with a single exponential component of exchange, and the data suggest a fully saturated Mg ATP complexing under these conditions

Reserpine in a concentration range between 2×10^{-8} and 2×10^{-7} M causes more pronounced [illegible]

This [illegible] stabilizing concentration range of reserpine causes no obvious deleterious effects on the vesicles at the electron microscopic level

An improved purification of isolated noradrenaline (NA) storage vesicles from bovine splenic nerve trunk has recently been described, including quantitative data on contaminating marker enzymes for the major subcellular components (Lagercrantz, Klein and Stjarne 1970) The fraction containing the highest sedimentable NA protein ratio was further subjected to electron microscopic analysis (Thureson Klein *et al* 1970, Klein and Thureson-Klein 1971) The pellet from this fraction was estimated to contain at least 40 % NA storage vesicles by volume

It was of interest to test NA exchange in this vesicle preparation A Millipore filter technique was employed which, by data analysis according to a closed two-compartment system (Solomon 1953), allowed estimation of unidirectional fluxes and the percentage of readily exchangeable vesicle NA The method also provided a means of detecting if NA fluxes had more than a single component

It is recognized that valuable information is available on NA exchange in oth fractions of nerve trunk vesicles (see Euler 1958, 1961, 1966 for refs) In some instai ces NA efflux rate from a vesicle fraction was estimated by continuous removal of N from the medium using potassium ferricyanide (Euler and Lishajko 1967)

The present communication describes a Millipore filter technique as applied to tl exchange of isotopically labelled NA in isolated adrenergic nerve trunk vesicles an includes analytical treatment of the data The major emphasis is on the effects of N concentration and reserpine inhibition in Mg ATP supplemented media

Methods

Preparation of isolated NA storage vesicles Bovine splenic nerve trunk was used to prepar isolated NA containing vesicles by a method similar to that previously described (Lagercrant Klein and Stjarne 1970 Klein and Lagercrantz 1971) For studies of H^3 *l* NA exchange i was desirable to achieve maximum vesicle yield from the sucrose heavy water density grad ent with minimum sacrifice of the high NA protein content It was also expedient to concentrat the vesicle fraction into a relatively small gradient volume This allowed direct dilution fron the gradient avoiding an intermediate sedimentation and resuspension which always resulte in some vesicle loss through membrane rupture Therefore the original gradients were mod fie so that fraction FIII and part of FII would be condensed into a narrow band This was ac complished by introducing a gradient "step at the bottom consisting of 1 5 ml of 1 3 M sucrose above which was placed 6 0 ml of the continuous sucrose heavy water gradient rangir from 0 25 M (top) to 0 6 M (bottom) There was space for 5 0—6 0 ml of crude vesicle suspension consisting of the 10 500 g—10 min supernatant from the homogenized nerve to be layered on the top These were centrifuged at 40 000 RPM for 75 min in a Beckman Mode L2 65B with SW40 rotor A dense band containing the NA vesicles formed at the interface of the continuous density gradient and the 1 3 M sucrose step The volume of the band was 1 0—1 2 ml of a total of 13 0 ml in the tube The modified FIII fraction thus formed is referred to as $FIII_M$ The $FIII_M$ was routinely diluted with constant mixing to 0 25—0 30 M sucrose containing 1 0 mM tris HCl buffer at pH 7 4 for use in isotope flux studies

NA was measured fluorometrically (Euler and Lishajko 1961) and prote n with the folin phenol reagent (Lowry *et al* 1951)

Millipore filter technique Samples of vesicle suspension were preincubated for 5 min under the desired conditions in an agitating water bath prior to the addition of H^3 I NA (New England Nuclear) One ml aliquots of labeled vesicles were volumetrically pipetted into Pyrex Millipore filter (MF) microanalysis apparati at various time intervals Celotate MF discs (EGWP 02500) with pore size 0 20 μ and 25 mm diameter were used routinely Several other pore sizes were tested for their applicability (see Results)

Filter discs were wet with distilled water and drained prior to admission of the vesicle suspension which was placed directly on the filter without suction applied This ensured homogeneous distribution of the suspension over the exposed filter disc surface Care was taken not to lose droplets of the sample on the side of the tube Suction was applied by means of a vacuum pump The pressure was adjusted so that the filtration step could be accomplished in less than 10 sec All parameters of the vesicle harvesting procedure were duplicated as nearly as possible from sample to sample The filtrate from each vesicle sample was collected in a clean tube fitted below the filter in the suction flask The MF disc with trapped vesicles was carefully removed placed d [illegible] 100 μl sample of each filtrate was also pl [illegible] ne absolute ethanol with fluors PPO 4 [illegible] a Packard Tri Carb automatic liquid [illegible] by internal standards

Correction for counts occluded in the MF discs and for counts displaced from the discs by trapped vesicles The following typical example is given for sample correction due to counts held in the MF discs Using a standard radioactive incubation medium with 100 000 cpm/ml and the other experimental conditions described the 0 20 μ Celotate disc retained 3300 cpm The content from one ml of the average non radioactive vesicle suspension ($\sim$0 07 mg protein/ml) filtered on the disc surface caused an average displacement of 9 % of the 3300 cpm Therefore about 3000 cpm would be subtracted from each MF disc sample containing filtered vesicles in order to arrive at the actual counts taken up by the vesicles This can be tested under any number of conditions which are known to prevent measurable NA exchange

the correction.

Due to the high concentration of NA in the vesicles compared to that in the medium and the rapid NA exchange under most conditions the correction was most significant at zero time and 2 min samples By 5 min the vesicles contained 2 or 3 times the counts occluded by the MF disc If potent inhibitors of exchange are employed, the correction can be an important factor during the entire exchange period The counts retained by the MF disc itself can be minimized by one or more rapid washes with identical non radioactive medium provided this also does not influence the vesicle counts A single 3 ml wash will reduce MF disc counts in the example above by about 75 % The washing procedure was not considered necessary to produce statistically accurate sample counts in the present study

Results

Test of NA particulate retention by MF discs A series of MF discs with rated pore sizes of 0 3, 0 22, 0 20 and 0 1 μ were tested for retention of sedimentable NA in 1 0 ml samples of typical vesicle suspensions MF discs of 0 22 and 0 20 μ pore size were about equally acceptable, giving retentions of >98 5 % of the sedimentable NA content The 0 3 μ disc allowed 5—10 % of the particulate NA to pass through, while the 0 1 μ disc increased the filtration time unduly under the conditions of our experiments Of the two acceptable filters, the Celotate 0 20 μ disc was used routinely as it was thinner and occluded less medium counts in the interspaces This resulted in a smaller correction factor i e, cpm in (MF disc+vesicles)—MF disc = cpm in vesicles

The average diameter of the isolated NA storage vesicles is 763 Å, range ~0 050 to 0 095 μ (Klein and Thureson Klein 1971) The fact that a filter disc pore diameter, which is 2—4 times the vesicle diameter, can retain essentially all of the vesicles is believed due primarily to the tortuous nature of the interspaces among the fibers in the disc Also, vesicles may tend to aggregate into small clusters The nearly complete retention by the filter further indicates that the NA, which is sedimentable at high gravitational forces, is nearly all associated with relatively large particles

Maintenance of particulate NA content during the experimental period All experimental media contained a final concentration of 3 or 5 mM Mg^{++} and ATP, and were supplemented at 15 min intervals with an additional 1 mM ATP to counteract any reduction due to hydrolysis The protective effect of periodic additions of ATP has been reported (Euler and Lishajko 1963) A final concentration of 10 μg ml Pargyline the monoamineoxidase inhibitor, was also routinely added in order to minimize inactivation of NA by possible mitochondrial contaminants The basic vesicle suspension medium contained, 20 mM tris HCl buffer, 50 mM sucrose, 100 mM K^+ and 10 mM Na^+, the latter mostly as chlorides The K^+ Na^+ ratio was used to approximate that naturally found in the neuroplasm The average $FIII_M$ fraction diluted for use in flux experiments contained 2 8 μg NA/mg protein, range 1 25 to 7 05 in 52 determinations The 20 % reduction in NA content compared to the original FIII fraction was an unavoidable result of the increased vesicle yield The NA content of the FIII fraction can be nearly doubled by a simple fractional centrifugation, when total vesicle yield is of little consequence (Klein and Lagercrantz 1971)

Vesicle samples were taken periodically during the experiments in order to follow any changes in NA and protein contents In 13 experiments at 20° C there was a negligible decrease of <3 % in the particulate NA protein ratio of samples during the usual 45 min experimental period with concentrations of 0 5 μg l NA/ml or greater In 18 experiments at 30° C there was a gradual 20 % decline in the NA protein ratio during the usual 45 min period with 0 5 μg l NA/ml When the concentration of NA in the medium was increased to 1 0 or 3 0 μg/ml (11 expts) the NA net loss decreased to 5 % or less The values above were not appreciably affected by 2×10^{-4} M reserpine (12 expts) Reserpine at 2×10^{-7} M prevented the decrease in particulate NA protein ratio with 0 5 μg NA/ml Although protein values are not available these findings are in general agreement with others in this laboratory under comparable conditions except for the use of isotonic potassium phosphate in the vesicle suspension media (Euler and Lishajko 1965 Euler, Stjarne and Lishajko 1963 1964 Stjarne 1964)

The observed H^3 l NA uptake by the vesicles was corrected proportionately for any gradual loss of particulate NA protein ratio due to vesicle ageing in the final calculations

General Transport Equation and analysis by a Closed Two Compartment System The exchange of H^3 l NA (*NA) is expressed by the General Transport Equation (equat. 1) Simply stated the amount of radioisotope in the vesicles at any time *t* is equal to the amount that enters (influx) minus the amount that leaves (efflux) k_1 and k_2 are the specific transfer coefficients for influx and efflux respectively $*NA_{vesicles}$ and $*NA_{medium}$ are cpm/ml in the respective compartments The latter should be directly proportional to the activities of NA but not necessarily to the total concentrations. For example a portion of the vesicle NA content may not be readily exchangeable (equat 3)

When a steady state is reached $t = \infty$ there is no net flux of isotope and $d{*NA_{vesicles}}/dt = 0$ The equation reduces to $k_1 \; {*NA_{medium}} = k_2 \; {*NA_{vesicles}}$ i e influx must equal efflux.

The following equations are used to analyse the NA exchange data from the MF technique described

$$(1)\quad d{*NA_{vesicles}}/dt = k_1 \; {*NA_{medium}} - k_2 \; {*NA_{vesicles}}$$

$$(2)\quad \text{Specific Activity } (SA) = \frac{\text{counts/min/ml}}{\text{meq NA/L}} = \text{counts/min/meq NA}$$

$$(3)\quad SA^{\infty}_{vesicles}/SA^{\infty}_{medium} \times 100 = \text{Percent Exchangeability}$$

$$(4)\quad \ln(1 - {*NA_{vesicles}}/{*NA^{\infty}_{vesicles}}) = -(k_1 + k_2)t$$

$$(5)\quad t_{1/2} = 0.693/k_1+k_2$$

$$(6)\quad {*NA^{\infty}_{vesicles}} = k_1 \; {*NA^{t=0}_{medium}}/k_1+k_2$$

$$(7)\quad {*NA^{\infty}_{vesicles}}/{*NA^{\infty}_{medium}} = k_1/k_2$$

$$(8)\quad \text{Influx} = \frac{k_1 \; [NA]_{medium}}{cm^2/L \text{ vesicles}} \quad \text{Efflux} = \frac{k_2 \; [NA]_{vesicles}}{cm^2/L \text{ vesicles}}$$

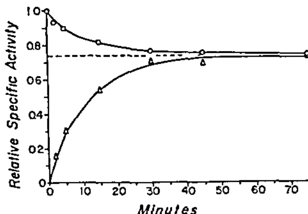


Fig 1 Relative Specific Activity of NA vesicles (△—△) and suspending medium (○—○) vs time Medium contained 0 5 μg *l* NA/ml, 3 or 5 mM Mg ATP, 100 mM K^+, 10 mM Na^+, 50 mM sucrose, 20 mM tris HCl buffer at pH 7 2—7 4 and at 30° C Samples were equilibrated 5 min prior to addition of tracer Dashed line is the estimated steady state An average of 17 experiments

Note that, in equations (1) through (4), $^*NA_{vesicles}$ and $^*NA_{medium}$ are in units of actual cpm/ml of vesicles and medium, respectively The *total vesicle volume* in any sample was estimated from the particulate protein content (~0 07 mg/ml sample), which was assumed to be 10 % of the wet weight, analogous to most biological systems

Relative specific activity curves in Mg ATP medium at 30° C When the SA of the medium and vesicles (equat 2) are plotted versus time, typical curves are obtained as shown in Fig 1 It is convenient to set $SA^{t=0}_{medium}$ equal to unity and to express all other points in relation to 1 0 This is the relative specific activity, RSA It can be seen that the medium loses and the vesicles gain *NA with time The curves approach a common steady state (dashed line) at about 40 to 50 min As the estimated steady state value $SA^{\infty}_{vesicles}$ equals SA^{∞}_{medium}, it indicates that the vesicle NA content is 100 % exchangeable (equat 3)

Graphical solution of —(k_1+k_2) and calculation of specific transfer coefficients According to equation (4), a semi logarithmic plot of the term at the left versus time will allow solution of the NA exchange curve for one or more components If it is described by a single exponential component, $^*NA = {^*NA^{t=0}} e^{-kt}$, it will be a straight line

In the present case, values for $RSA_{vesicles}$ (lower curve, Fig 1) were subtracted from $RSA^{\infty}_{vesicles}$ (dashed line, Fig 1) at various times, and the differences plotted on a semi logarithmic scale (Fig 2) The slope of the line (Λo /sec) equals —(k_1+k_2) and is calculated using equation (5) from its half life, which is determined graphically

The specific transfer coefficients, k_1 for influx and k_2 for efflux, are calculated by substitution into equations (6) and (7), respectively

Effect of NA concentration and temperature on unidirectional NA transfer coefficients The unidirectional transfer coefficients give a measure of the relative permeability of the vesicle membrane to NA in both directions The effect of NA concentration in the medium between ~0 5 and 3 0 μg/ml is shown in Fig 3A for influx and in Fig 3B for efflux At 30° C in Mg ATP medium, influx transfer cofficients are inversely related

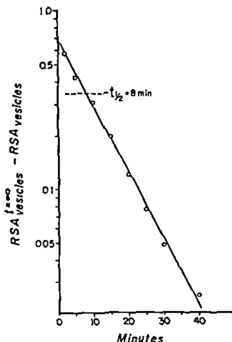


Fig 2 Data from Fig 1 Semi logarithmic plot of differences in the relative specific activity (RSA) of the vesicles at various times and the RSA at the steady state $t_{1/2}$ = half life

to NA concentration Conversely, efflux transfer coefficients are directly related to NA concentration in the medium At the lower NA concentrations, the Q_{10} for k_1 etween 20 and 30° C is about 2 8 and for k_2 it is about 2 4

The relative permeability of the vesicles for NA entry is 650 times that for efflux at 0 5 μg NA/ml This ratio is reduced to 106 when NA in the medium is increased to 3 0 μg/ml (Table I)

Absolute NA flux rates Absolute flux rates are expressed as picaMoles per second

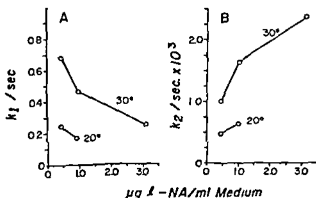


Fig 3 Specific transfer coefficients for unidirectional NA movements across the vesicle membrane A influx k_1 B efflux k_2 at 30 and 20° C vs NA concentration in the medium Medium content as in Fig 1 Average of 3—6 experiments at each point except data from Fig 1 is 17 experiments The differences between means at the same temperature and between pairs of means at the different temperatures are all statistically significant at the -$T_{.01}$ level

reserpine are indicated as approximations only, as there were statistical problems with the isotope data at this level of NA exchange

Measurement	μg l NA per ml Medium								
	0 5			1 0			3 0		
	Control	Reserpine		Control	Reserpine		Control	Reserpine	
		2×10^{-8} M	2×10^{-7} M		2×10^{-8} M	2×10^{-7} M		2×10^{-8} M	2×10^{-7} M
k_1	0 69	0 51	~0 002	0 46	0 31	0 006	0 25	0 24	0 08
$k_2\times10^3$	1 06	0 93	~0 14	1 63	1 16	0 33	2 36	2 30	1 13
k_1/k_2	650	544	~14	355	267	19	106	104	71
Absolute Flux Rate pMoles $\times10^3$ sec^{-1} cm^{-2}									
30°	2 26	1 62	~0 005	3 43	2.29	0 03	5 75	5 68	1 45
20°	0 87			1 18					

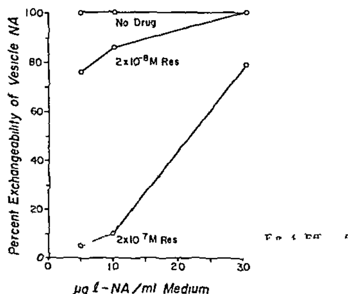
across one square centimeter of vesicle surface (pMoles sec^{-1} cm^{-2}) Using an average diameter of 750Å and treating the NA vesicle as a sphere, the volume $= 2\ 21\times10^{-16}$ cm^3/vesicle and the surface area $= 1\ 77\times10^{-10}$ cm^2/vesicle There is a total surface area of $8\ 0\times10^6$ cm^2/L vesicles

In the Mg ATP medium at 30° C, the average of 17 experiments gave a concentration of 2 89 μg NA/mg vesicle protein Assuming protein equals 10 % of the fresh weight, the vesicles contained 1 71 mMolar NA The actual content of 100 % pure vesicles is estimated to be about 3 times this (Klein and Thureson Klein, 1971) By substituting the concentration values for NA_{medium} and $NA_{vesicles}$ (exchangeable fraction) into equations (8), the absolute flux rates can be determined

In the steady state the absolute flux rate is $2\ 26\times10^{-3}$ pMoles sec^{-1} cm^{-2} at 30° C with 0 5 μg NA/ml in Mg ATP medium Absolute flux rates at various NA concentrations and with reserpine present are given in Table I The rate of NA exchange or flux in the vesicles is directly proportional to the concentration of NA in the medium The Q_{10} between 20 and 30° C is 2 5—3 0 for the lower range of NA concentration

Effects of reserpine on NA transfer coefficients and vesicle NA exchangeability Unidirectional transfer coefficients, k_1 k_2 ratios and absolute fluxes are given in Table I Percent exchangeability is given in Fig 4 Two concentrations of reserpine were chosen to demonstrate their effects on vesicle membrane permeability to NA One drug concentration caused partial inhibition and the other nearly complete inhibition

With 0 5 μg NA/ml, the ratio of k_1 k_2 is markedly reduced in the presence of 2×10^{-7} M reserpine The influx coefficient, k_1 is actually diminished by a factor of 345, while k_2 is diminished only by a factor of 7 or 8 The percent exchangeability of



vesicle NA is reduced to a barely significant level, ~5 % Thus, the effective driving force for efflux (exchangeable vesicle NA) is nearly eliminated by 2×10^{-7} M drug Even at 2×10^{-8} M reserpine the exchangeability is reduced 24 % and there is a significant but lesser inhibitory effect on the k_1 k_2 ratio

A similar pattern emerges at 1 0 μg NA/ml in the medium, except that the inhibitory effects are reduced In fact, as the NA concentration is raised in the medium to 3 0 μg/ml the effect of 2×10^{-8} M reserpine is no longer observed Even at 2×10^{-7} M drug which for all practical purposes inhibited NA flux completely at 0 5 and 1 0 μg NA ml there is a strong reversal of the inhibition The exchangeable fraction of vesicle NA rose to nearly 80 % The relative inhibition of inward NA permeability compared to outward permeability was only 30 % from the control value However k_1 itself was reduced some 70 % and k_2 about 50 %, resulting in a 75 % decrease in flux rate compared to the control

Effect of reserpine on the ultrastructure of isolated nerve trunk vesicles Homogeneous populations of isolated NA storage vesicles from bovine splenic nerve trunk have been identified electron microscopically (Thureson Klein *et al* 1970, Klein and Thureson Klein 1971) The vesicles can be produced in a relatively large and very pure layer at the top of an FIII fraction pellet They can be filled and emptied of their intravesicular matrix material under conditions identical to those know … produce part… results in NA content

Similar FIII and further purified $FIII_M$ … (Klein and T… 1971) ves… fractions were treated with 5×10^{-8} M reserpine The typi… appearance in the Mg ATP medium with and witl… Fig… There is no obvious detrimental effect of reserpin…

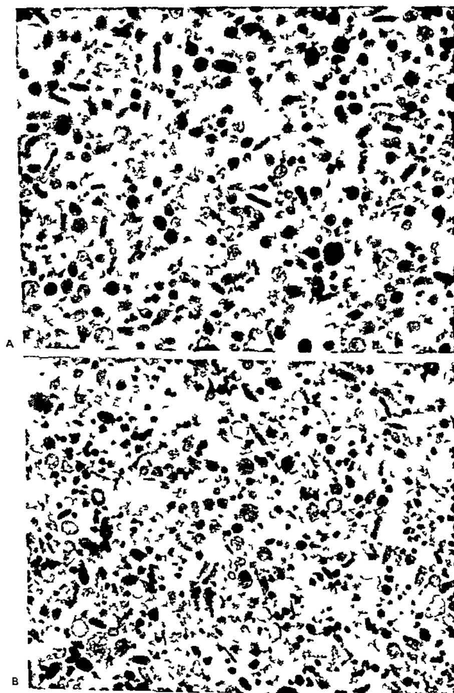


Fig 5 El c on m crographs of ves les n the large relat ely pure upper la er of h pe
C ra d h d

Discussion

An improved preparation of NA storage vesicles isolated from bovine splenic nerve trunk has been tested for unidirectional fluxes of H^3/NA using a Millipore filter technique

In a Mg ATP supplemented medium at 30 and 20 °C, the vesicle NA content is completely exchangeable with NA in the medium (Fig 4) This is true over a range of 0 5 to 3 0 µg NA/ml at 30° C, and there is no reason to believe it does not hold also for higher concentrations and other temperatures Using a different isotope method, isolated splenic nerve trunk vesicles were reported to exchange 90 % of their NA content in 20 min and all in 40 min at 37° C, in a medium containing 1 5 × 10⁻⁶ M NA and Mg ATP (Schumann and Burger 1970) Although our results at 30° C would indicate a more rapid exchange at 37° C than that reported in the latter study the results on exchangeability are generally in good agreement However, it is difficult to understand the finding that after equilibrium with C^{14} NA at 37° C in the presence of the above medium, a second incubation for 30 min at 20° C with unlabelled NA at 2 5 × 10⁻⁶ M resulted in no statistical loss of tracer from the vesicles (Schumann and Burger 1970) Our data gives a 19 5 min half life for NA exchange at 20° C in a comparable medium (0 5 µg NA/ml) which means that more than 50 % of the total label should easily exchange in a 30 min period Of course, the actual loss of vesicle label to a new equilibrium with an unlabelled suspension medium would be in part governed by the volume ratio of vesicle and extravesicular compartments

The Q_{10} s for influx and efflux transfer coefficients and for absolute flux are in the 2 4 – 3 0 range (Fig 3 and Table I) This suggests some sort of metabolic dependent process controlling both the influx and efflux of the transmitter It would be incredulous to suggest that both NA entry and exit in the vesicles be governed by an active membrane transport In addition there is no evidence for a link between NA transport with ATP hydrolysis in these vesicles (Klein and Lagercrantz 1971) The authors favor the idea that the Q_{10} is characteristic for the binding unbinding process of NA from its intravesicular complex with which the vesicle membrane may be an integral part

It is interesting to find that as the driving force for influx is increased over the range of 0 5 to 3 0 µg NA ml of medium the relative permeability for NA entry (k_1) *becomes less while that for NA exit (k_2) becomes greater (Fig 3) These changes are* such that the absolute flux of NA in each direction is maintained in balance with minimal or no net change in NA content of the vesicles It suggests that there is a nearly fully saturated Mg ATP dependent complexing of NA in the vesicle matrix under these conditions One can force moderate additional NA uptake using higher medium NA concentrations but this is believed to be primarily the result of a much increased passive inward driving force not specific for Mg ATP

A previous estimation of NA outflux has been made using $K_4Fe(CN)_6$ in the medium to cause continuous removal of NA (Euler and Lishajko 1967) This resulted in a doubling of their "release rate" found in earlier observations with a half life of

35—40 min This was in the presence of $1-3 \times 10^{-6}$ M NA and at 20° C, but without addition of ATP Their "release constant" is not strictly comparable with the present efflux transfer coefficient, k_2 because the former is an estimate under non steady state conditions That is, there was a continuous net loss of vesicle NA, both because ATP was omitted and because NA was removed by the ferricyanide Without a correction relating to the kinetics of this net loss, true efflux rates cannot be calculated Such a correction would tend to increase the half life On the other hand, the presence of ATP would tend to increase the rate of NA exchange and shorten the half life In the present paper using a K^+ and Na^+ salt combination in lieu of isotonic potassium phosphate medium and with ATP present, the half life for *NA efflux was ~23 min at 20° C We have tested NA exchange by the Millipore filter technique in an isotonic potassium phosphate medium under steady state conditions for comparison purposes The results show some slowing of the exchange rate of NA compared to that in the suspension medium used in this study

The data also show that the exchange of vesicle NA is described by a single exponential component This indicates that vesicle NA is essentially all in a single pool One cannot absolutely exclude the possible existence of a second pool of exchangeable NA in the vesicles However, if a second pool exists, its size and rate of exchange are such that any influence compared to the main pool is not readily evident The single 100 % exchangeable NA pool in splenic nerve trunk vesicles may be contrasted to the relatively small exchangeable pools and large not readily exchangeable pool of catecholamines reported for bovine adrenal medullary vesicles (Taugner and Hasselbach 1966)

The application of the unidirectional flux method to determine the effects of reserpine were particularly informative It was pointed out previously in a study of the effects of reserpine on vesicle ATP hydrolysis that it is important to study the effects of the drug in a reasonably 'therapeutic concentration range (Klein and Lagercrantz, 1971) High NA depleting drug concentrations cause a marked loss of NA from isolated vesicles through irreversible effects on membrane integrity, leading to subsequent rupture of the vesicles Thus any attempts to measure NA exchange rates under these conditions would be meaningless At 5×10^{-6} M reserpine, there is no evidence of vesicle damage at the electron microscopic level (Fig 5) In fact it may be that the intravesicular matrix has a sharper contrasting fine granular appearance than in the absence of drug This finding is not entirley unexpected because the vesicle membrane stabilizing effect of the drug at this concentration actually depresses the slow loss of NA which occurs even with Mg ATP and 0 5 μg NA/ml present

The inhibitory effects of reserpine in the 2×10^{-8} to 2×10^{-7} M range are more pronounced on relative inward NA permeability (k_1) than on outward permeability (k_2) These conditions would normally cause a rapid loss of vesicle NA content except that the drug also inactivates a proportional amount of the readily exchangeable vesicle NA pool This results in a decrease in the effective NA outward driving force, and although the absolute flux can be reduced by a factor of 100 or more (Table I),

medullary blood flow, and Barton *et al.* (1968) observed a disproportionate decrease in blood flow to the superficial cortex when perfusion pressure was reduced However, other studies have shown autoregulation of both medullary (Aukland 1966 Grangsjö 1968, Wolgast 1968, Stinson *et al* 1969) and cortical blood flow (Grangsjö 1968, Wolgast 1968, Stinson *et al* 1969). The purpose of the present experiments was to reinvestigate this problem, using hydrogen gas clearance as an indicator of local blood flow and an electromagnetic flowmeter to measure total renal blood flow At stepwise reductions in renal perfusion pressure by means of a clamp on the aorta or the renal artery, autoregulation of blood flow to superficial and deep layers of cortex and to outer medulla was examined in anesthetized dogs The pressure flow relationship of outer medulla was also examined after atropinization, as cholinergic mechanisms are believed to be involved in the autoregulation of renal medullary blood flow (Stinson *et al* 1969)

Methods

... kidney was investigated in 18 mongrel ... food was withheld for 19 hours before ... nimals were anesthetized with sodium ... was maintained with additional doses ... and catheters ... ions, ... right ... care being taken to avoid damage to the renal nerves A polyvinyl tube of 0 4 mm external diameter was inserted in upstream direction into the renal artery ad modum Herd and Barger (1964) for injection of hydrogen saturated saline and, in most animals, for recording mean renal arterial pressure ($\overline{RAP}$) Mean arterial pressures were measured with Statham transducers and recorded continuously on a Sanborn recorder In most experiments, $\overline{RAP}$ was reduced by means of an adjustable clamp on the renal artery proximal to the tip of the polyvinyl tube In seven dogs aorta was constricted above the origin of the renal arteries with a Blalock clamp ... in the femoral artery In three ... which was the average differ... th were measured Mean total ... tromagnetic flowmeter (Nycotron Oslo and recorded on a Sanborn recorder A flowmeter probe, 3—4 mm in diameter depending on vessel size was applied to the renal artery in 16 dogs, proximal to the clamp. Calibration of the probe had been performed previously on femoral and carotid arteries in other experiments Zero flow was checked by occluding the renal artery for 5 to 10 sec

Hydrogen clearance was used as indicator for local blood flow (Aukland, Bower and Berliner 1964, Aukland 1968) Local hydrogen concentration was measured with platinum electrodes and a 6-channel polarograph and recorded on a Rikadenki 6-channel recorder (Rikadenki Co, Tokyo, model B64), using a polarizing potential versus a KCl saturated calomel reference electrode of +0 2 V Clearance per volume tissue was calculated from the hydrogen desaturation curves, as their initial rate constant (Aukland 1968) For measurements in outer medulla H_2 gas was administered both by inhalation and by injection of 10—20 ml H_2 saline as described by Aukland and Loyning (1970) In the cortex a stable tissue concentration was obtained by intra-arterial injection alone Cortical and medullary blood flow were measured in separate groups, as the much higher clearance from the renal cortex permitted more frequent clearance measurements

... 2—3 and 6—7 mm into the renal cortex three of the L-shaped electrodes measured 0 5—1 0 ... In 11 dogs, 6 L-shaped platinum electrodes ... kidney tentatively into the outer medulla, the ... tapered tips measuring 1 0—1 5 mm in length and 0 3 mm in largest diameter The external

part of the electrode was stitched to the capsule, the kidney replaced in its normal position, and the wound temporarily closed

Measurements were started about half an hour after completion of the surgical procedure After two control periods, renal arterial pressure was reduced in one or two steps down to the lowest autoregulating pressure level (controlled by observing total blood flow), and usually also to a pressure below this level

To reduce or [illegible]
(Stinson *et al*
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choline injected into the renal artery A second sequence of renal arterial constriction was then performed

At the end of the experiments, the renal vessels were clamped, and the kidney removed to check the positions of the electrodes Results were discarded if the electrodes were macroscopically in contact with calyces or connective tissue The kidney was drained before weighing Because of uncertainties as to blood flow per gram of tissue in the different compartments of the kidney, as estimated from total blood flow and kidney weight, changes in total and local blood flow during pressure reduction were compared for statistical evaluation, whereas paired comparison was made between absolute values of local blood flow in the superficial and deep layers of cortex

Any change in kidney volume will influence hydrogen clearance which is determined by blood flow per volume tissue In 4 dogs, therefore, changes in cortical and outer medullary volume during reduction in perfusion pressure were estimated by ultrasonic measurement of intrarenal distances Piezo-electric crystals, 1 × 2 mm in size, were implanted in the cortex and medulla and radial distances recorded as described by Kiil *et al* (1971)

Results

Cortical blood flow Hydrogen concentration recorded with one electrode in the outer cortex before and during reduced perfusion pressure is shown in Fig 1 (H_2-concentration as electrode current in arbitrary units) A steady tissue-blood concentration of H_2 was obtained within approximately 45 sec and maintained for another 30 sec. After stopping infusion of H_2-saturated saline (arrow), a delay of 2—4 sec was observed before the desaturation took a monoexponential course, with no significant deviation until desaturation was more than 90 % complete In control periods (Fig 1, I and II) the desaturation curves were practically identical When perfusion pressure was reduced, cortical H_2-clearance parallelled total blood flow, being fairly constant down to 85 mm Hg and markedly reduced below that pressure

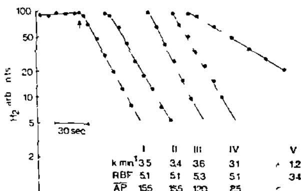


Fig 1 *Semilogarithmic plot of hydrogen desaturation curves from one electrode in outer cortex in control (I and II) and during reduced renal perfusion pressure (III to V)* I_{H_2} = hydrogen oxidation current in arbitrary units, k = desaturation rate constant in min⁻¹ RBF total renal blood flow in ml/min g kidney weight, $\overline{AP}$ = mean aortic blood pressure in mm Hg

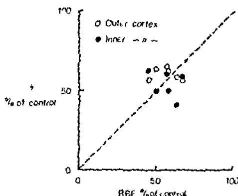


Fig 2

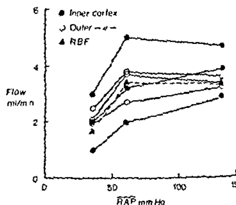


Fig 3

Fig 2 Relationship between H_2 clearance in the cortex (k) and total renal blood flow (RBF) both in per cent of control at renal perfusion pressures below the level of RBF autoregulation (Each point average of 2—3 electrodes)

Fig 3 Blood flow measured in outer and inner cortex as hydrogen gas desaturation rate (flow ml/min ml = k, ml^{-1}) and in the renal artery with an electromagnetic flowmeter (RBF ml/min g) both in relation to mean renal arterial blood pressure ($\overline{RAP}$, mm Hg) (O, ●, ▲ measurements from individual electrodes)

level Similar results were obtained with all cortical electrodes, both in the same animal and in different dogs

As evident from Fig 2, a similar decrease in RBF and H_2-clearance was obtained when renal arterial pressure was greatly reduced (average 38 mm Hg range 30—50 mm Hg) RBF decreased to an average of 57 % of control, and H_2-clearance from outer and inner cortex to 61 % and 54 % of control respectively The difference in total and local blood flow changes, as measured with electromagnetic flowmeter and H_2-clearance respectively, was not significant at the 5 % level

Fig 3 shows the results in one dog with three electrodes in outer cortex and three in inner cortex Blood pressure was reduced in one step from 135 mm Hg to 60 mm Hg, with no change in total blood flow Two electrodes in outer cortex and one in inner cortex showed excellent autoregulation of local blood flow, and of the same degree as RBF Although not to the same extent the other 3 electrodes also showed autoregulation of local blood flow Similar results obtained in 6 other dogs are summarized in Table I, in which local blood flow in outer (OCF) and inner (ICF) cortex is given as the average of 2—3 electrode measurements in each region At control pressure OCF and ICF averaged 3 26 ml/min g and 3 45 ml/min g respectively the difference between them being insignificant at the 5 % level Fig 4 shows, in per cent of control, the changes of total blood flow (upper panel) and of blood flow in outer and inner layers of cortex (middle and lower panels) related to perfusion pressure, in mm Hg The suggested l[illegible] RBF variations in the upper panel were transferred to the middle and l[illegible] better comparison with local blood flow changes A[illegible] 1 mm Hg to 76 mm

TABLE I Effect of reduction in renal perfusion pressure on total and local renal blood flow in 7 dogs

	$\overline{AP}$ mm Hg	$\overline{RAP}$ mm Hg	RBF ml/min g	OCF ml/min g	ICF ml/min g
Dog 1	145		3 8	3 7	3 0
	115		3 6	3 3	3 0
	95		3 6	3 1	2 7
	55		1 7	2 1	1 9
Dog 2	155		5 1	3 5	3 4
	120		5 3	3 6	3 5
	85		5 1	3 3	3 2
	60		3 4	2 0	2 0
Dog 3	140		4 3	3 9	
	100		4 3	3 0	
	70		3 0	2 5	
Dog 4	150		2 7	2.2	3 2
	110		2 7	2 0	2 6
	95		2 7	1 9	2 4
	45		1 7	1 3	1 3
Dog 5	145	145	3 0	3 1	4 3
	140	70	3 0	3 0	3 9
	140	30	1 7	2 0	2 7
Dog 6	135	125	3 4	3 5	3 8
	140	60	3 5	3 4	3.2
	140	35	1 7	2 2	2 0
Dog 7	105	105	4 0	2 9	3 0
	115	75	3 8	2.2	2 8
	110	65	3 8	2 6	2 7
	115	35	2 3	1 9	1 5

$\overline{AP}$ = mean aortic pressure, $\overline{RAP}$ = mean renal arterial pressure, RBF = mean total blood flow per gram kidney weight OCF = outer cortical blood flow ICF = inner cortical blood flow (local blood flow as mean rate constant of H_2-clearance from 2—3 electrodes in outer and inner cortex)

TABLE II Effect of reduction in renal perfusion pressure on total and local renal blood flow in one dog before and after atropine infusion

Time min	Experimental condition	$\overline{RAP}$	RBF ml/min g	Outer medullary H_2 clearance E_1 min^{-1}	E_2 min^{-1}	E_3 min^{-1}
0	Control	135	5 4	0 86	0 91	0 68
30	Control	135	6 0	0 78	0 80	0 67
55	Constriction	95	6 0	0 65	0 78	0 61
83	Control	140	6 1	0 78	0 87	0 61
95	Atropine 0 5 mg/kg b w (11 mg i v)					
130	Control	140	6 0	0 80	0 80	0 68
140	Constriction	85	6 0	0 67	0 71	0 67
165	Control	135	5 6	0 75	0 75	0 64

Kidney weight 53 g
E_1 E_2 E_3 = measurements from individual electrodes $\overline{RAP}$ and RBF as in Table I

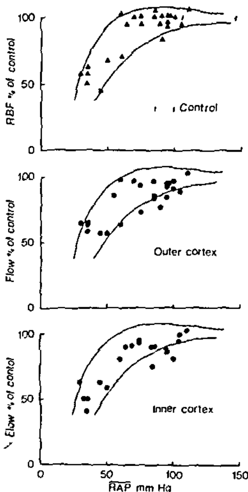


Fig 4

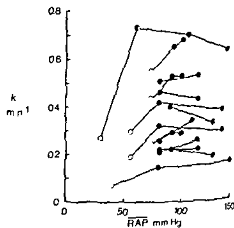


Fig 5

Fig 4 *Total blood flow (RBF* upper panel) and flow in outer and inner parts of cortex (middle and lower panels respectively) all in per cent of control in relation to mean renal arterial blood pressure ($\overline{RAP}$ in mm Hg) For explanation see text

Fig 5 Local blood flow in outer medulla (k min⁻¹) (measurements from individual electrodes) in relation to mean renal arterial blood pressure ($\overline{RAP}$ mm Hg) Closed circles RBF unchanged (<10 %) open circles RBF reduced (>10 %)

Hg did not reduce RBF by more than 1 % At this pressure level, a mean decrease in local blood flow to 89 % of control was observed in outer cortex and to 87 % of control in inner cortex The difference between OCF and ICF was not significant, whereas the difference between local and total blood flow within the autoregulating pressure range (down to 70 mm Hg) was significant at the 5 % level

Outer medullary blood flow The protocol of one experiment is shown in Table II After two control periods one step of pressure reduction from 135 mm Hg to 95 mm Hg, was performed At this pressure level, total renal blood flow was un

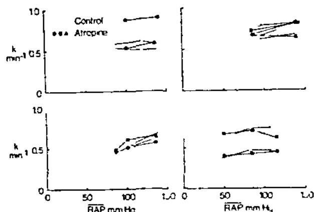


Fig 6 Individual electrode measurements of outer medullary blood flow (k, min⁻¹) before (○□△) and after (●■▲) atropine infusion in relation to mean renal arterial blood pressure ($\overline{RAP}$, mm Hg)

changed — whereas outer medullary blood flow decreased on average to 91 % of control After release of the clamp, atropine was given i v in doses abolishing the vasodilator effect of 4 μg acetylcholine injected into the renal artery Control RBF and H_2 clearance before and after atropine were unchanged and RBF also remained unchanged down to 85 mm Hg, whereas medullary blood flow was reduced on average to 92 % of control

H-clearance in relation to renal arterial blood pressure obtained with 23 electrodes in 11 dogs is summarized in Fig 5 and 6 At 16 electrode sites, pressure reductions within the range of total blood flow autoregulation (decrease in RBF less than 10 %) caused almost no fall in local blood flow At the remaining 7 electrode sites local flow fell, but proportionally less than $\overline{RAP}$ Reduction of $\overline{RAP}$ to an average of 50 mm Hg effected a 40 % decrease in H-clearance (Fig 5, open circles), similar to that observed for RBF Atropine did not influence total blood flow, the average being 4 7 ml/min g before and after atropine administration After atropinization (Fig 6), at a reduction in perfusion pressure similar to that in control experiments reduced blood flow was recorded by one of 9 electrodes (Fig 6 lower left) whereas 8 electrodes showed unchanged or increased medullary blood flow The average change in MBF was 94 % of control both before and after atropine infusion at an average reduction in $\overline{RAP}$ to 92 mm Hg, RBF was 94 and 100 % respectively

Summarizing the results on renal medullary circulation Fig 7 shows total blood flow (RBF, upper panel) and outer medullary blood flow (MBF lower panel both in per cent of control, related to renal perfusion pressure ($\overline{RAP}$ in mm Hg The suggested limits of RBF variations during clamping (upper panel) were transferred to the lower panel for comparison with changes in MBF At an average fall in $\overline{RAP}$ from 126 mm Hg to 88 mm Hg RBF decreased by 4 % whereas on average no change was observed in MBF (range 81—116 %) The mean difference between MBF and RBF, both in per cent of control did not differ significantly from zero

The effect of reduced perfusion pressure on intrarenal distances is summarized in

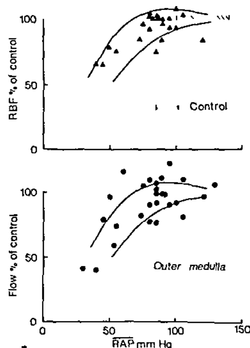


Fig 7 Total blood flow (RBF, upper panel) and flow in outer medulla, both in per cent of control, in relation to mean renal arterial blood pressure ($\overline{RAP}$, in mm Hg) For explanation, see text

TABLE III Effect of reduced perfusion pressure on intrarenal distances

Dog no	AP mm Hg	RBF ml/min	% of control	CD mm	% of control	MD mm	% of control
I	115	155		9 40		10 45	
	75	155	100	9 30	99	10 30	99
	60	150	97	9 30	99	10 13	97
	45	121	88	9 00	96	9 50	92
II	130	160		10 25		9 15	
	95	168	105	10 15	99	9 15	100
	75	168	105	9 95	97	ns	
III	125	290		6 00		12 30	
	80	285	99	6 00	100	12 00	98
	60	245	85	5 80	97	11 60	94
IV	140	125		7 52		9 44	
	85	115	92	7 50	99 5	9 38	99
	65	100	80	7 40	98	9 40	99 5

AP = aortic pressure, RBF = total renal blood flow, CD = cortical distance, MD = medullary distance
ns = no signal

Table III At an average reduction of aortic blood pressure to 65 mm Hg (range 60—75 mm Hg) distances in both cortex and medulla were reduced by 2 %, on average (range 0 5—3 %), indicating a volume decrease of approximately 6 % Total renal blood flow fell to 91 % of control at this pressure level (range 80—105)

Discussion

The relationship observed in the present experiments between renal arterial blood pressure and H_2-clearance from different parts of the renal cortex and outer medulla indicates that blood flow is well autoregulated in all regions

Clearance of H_2 as indicator of local blood flow has been extensively studied previously (Aukland *et al* 1964, Neely *et al* 1965, Aukland 1968), and good correlation to ^{85}Kr clearance and erythrocyte transit time has been demonstrated (Aukland and Wolgast 1968) By excluding other factors influencing clearance of inert gas H_2-clearance has been considered a function of blood flow in the area around the electrode Recordings of hydrogen gas clearance from the renal cortex made by Aukland *et al* (1964), however, were difficult to interpret because of multiexponential desaturation curves at the majority of the electrode sites Most of the electrodes in the present investigation gave monoexponential desaturation curves with hardly any significant deviation until 90 % desaturation (Fig 1) This marked improvement relative to the previous recordings probably results from the use of smaller electrodes fixed to the renal capsule, and to the replacement of the kidney in its original bed with the wound closed during measurements

Great variations in total renal blood flow are observed with no direct relation to kidney weight There may be no discrepancy therefore, between the higher average cortical blood flow observed by Wolgast (1968) using semiconductor detectors and P^{32} labelled red cells, and the present results obtained with H_2-clearance In the same animal, good correlation existed between the two methods (Aukland and Wolgast 1968)

The absolute blood flow estimated from total renal blood flow and kidney weight was on average some 8 to 12 % higher than cortical blood flow estimated from cortical electrodes Arteriovenous shunts might explain such a difference but no direct evidence for such shunts has been presented The difference cannot be accounted for by perfusion to the renal medulla or the renal capsule since blood flow per volume tissue in these compartments is lower than cortical blood flow A reduction in blood flow to the area measured due to damage of the tissue caused by insertion of the electrode might occur to some extent

Diffusion exchange of hydrogen gas with the renal surroundings is probably of no significance since all electrodes were placed at least 1 mm beneath the renal surface The similar percentual reductions in total blood flow and cortical clearance observed when perfusion pressure was reduced well below the level of blood flow autoregulation (Fig 2) indicate that H_2-saturation curves give valid estimates of local cortical blood flow

Due to countercurrent exchange H_2 gas clearance from the medulla may vary as an exponential function of blood flow, the exponent being a number between 1 and 2 (Berliner *et al* 1958 Thurau and Deetjen 1962) Hence, a change in blood flow should cause a more than proportionate change in clearance The lack of significant difference between percentual changes in *RBF* and *H* clearance in medulla therefore suggests a very efficiently operating autoregulating mechanism keeping medullary blood flow constant within the pressure range of total blood flow autoregulation

In the cortex countercurrent exchange of gas might also be expected between interlobular arteries and veins similar to the gas shunt proposed in skeletal muscle circulation (Aukland, Akre and Leraand 1967) A possible shunt would increase with decreasing blood flow and mainly affect outer cortex, causing a larger decrease in clearance in this region with reduction of perfusion pressure However no significant difference was found between outer and inner cortical clearance in control or during pressure reduction suggesting that shunting of gas between interlobular vessels has little influence upon clearance in the cortex and that autoregulation of local blood is equally efficient in superficial and deep cortex as indicated in Fig 3

Greatly reduced renal perfusion pressure causes the kidney to shrink The rate constant of H_2 desaturation indicating blood flow per volume tissue (ml/min ml) would thus be too highly estimated when compared to total blood flow through cortex or medulla Volume reductions in cortex and medulla showed an average decrease of 6 % (range 2—9 %) at a mean reduction to 65 mm Hg (range 60—75 mm Hg) A downward correction of cortical and medullary flow by 6 % would not alter the conclusion that local blood flow is autoregulated

Since the major part of blood flowing through the renal artery perfuses the cortex it is obvious that autoregulation of total renal blood flow must imply autoregulated flow in the whole or major part of the cortex This hypothesis has also been confirmed by Wolgast (1968) and Grangsjo (1968) by local or regional measurements However as those investigators did not make any distinction between the different regions the possibility still existed that some regions of the cortex might show less efficient autoregulation than others as suggested by Barton *et al* (1968) With a H-clearance technique similar to that described in the present experiments they observed that deep cortical flow exceeded superficial flow and that reductions in perfusion pressure caused a disproportionate fall in blood flow to outer cortex Their observations were not confirmed by the present studies which showed no difference between average blood flow in the outer and inner halves of the cortex and the same extent of autoregulation in the two regions The reason for the discrepancy is not clear but could reside in different experimental conditions However, Barton *et al* (1968) did not detail methods in their preliminary report

Using an external counting technique involving measurement of the transit times for ^{85}Kr and ^{131}I labelled albumin rapidly injected into the renal artery Munck *et al* (1970 studied the cortical distribution of blood flow before and during

saline loading Blood flow per volume tissue in a 1 mm thick layer of cortex under the renal capsule was only about half the blood flow observed in cortex as a whole The average blood flow obtained however is influenced proportionately more by blood flow in the superficial layer of the 1 mm thick mass of cortical tissue (Ingvar and Lassen 1962) The contribution of blood flow to the renal capsule is therefore difficult to estimate as is the effect of surgery—particularly exposure of the kidney—on blood flow within and just beneath the renal capsule

The present observation that outer medullary blood flow is autoregulated agrees well with preliminary results obtained by Aukland (1966) using a similar technique, and by Grangsjo (1968) and Wolgast (1968) with their indicator dilution technique (heat and P^{32}, respectively) On the basis of dye transit time, Thurau *et al* (1960) concluded that inner medullary circulation does not show the pressure flow relationship of autoregulation Study of their data however, reveals some degree of autoregulation in 9 of 11 cases (Aukland 1966) Girndt and Ochwadt (1969) found increased rate of uptake of ^{86}Rb in the medulla of the control kidney of rats made hypertensive by means of a clamp on the artery of the other kidney 4 weeks earlier They concluded that the increase in blood flow was due to lack of autoregulation of medullary blood flow, although mechanisms other than blood flow autoregulation seen during acute variations in perfusion pressure may account for their observations

Autoregulation implies regulation independent of renal nerves Stinson *et al* (1969) suggested that intrarenal cholinergic ganglia may be involved in autoregulation of medullary blood flow because they found reduced uptake and washout rate ^{85}Kr in juxtamedullary and medullary tissue during reduced perfusion pressure after atropine infusion It might be queried however whether their method can distinguish clearly between cortical and medullary blood flow In the present experiments in which local blood flow was measured with electrodes in the tissue atropine in doses that abolished or greatly reduced the effect of acetylcholine on renal blood flow did not abolish autoregulation of outer medullary blood flow suggesting that cholinergic mechanisms are not involved (Fig 5)

In spite of unchanged RBF and GFR a linear relationship exists between sodium excretion and renal perfusion pressure (Selkurt 1951) The observed autoregulation of local blood flow throughout the cortex and in outer medulla excludes a linear correlation between cortical or medullary blood flow and sodium excretion within the autoregulation pressure range Redistribution of blood flow in the kidney cannot therefore explain the reduction in sodium excretion when perfusion pressure is reduced at constant RBF and GFR

Mean difference between percentual changes in RBF per gram kidney weight and H clearance during pressure reduction within the range of autoregulation was 11 % and 8 % for outer and inner cortex respectively—both significantly different from zero ($p < 0.05$) Opening of arterio venous (a v) shunts when renal arterial pressure is reduced might account for this difference but no direct evidence of a v shunts in the kidney has so far been presented A more likely explanation is that the insertion of electrodes interferes to some extent with the autoregulation in immediate

surrounding tissue autoregulation continues to dominate, but its capacity is less th in undisturbed areas as indicated by total renal blood flow autoregulation In fav of this view is the excellent autoregulation of medullary blood flow, the lateral sertion of electrodes into the outer medulla will not disturb the juxtamedull arterioles which probably regulate blood flow to this region

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Acta physiol scand 1971, 83 203—209

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Efferent Impulses in the Chorda Tympani Nerve of the Rat

By

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Abstract

HELLEKANT, G *Efferent impulses in the chorda tympani of the rat* Acta physiol scand 1971 *83* 203—209

Effe [illegible] neuroleptic [illegible] d from the [illegible] ie combined [illegible] tructures in side the tongue

It is well known that electrical stimulation of the peripheral part of the cut chorda tympani nerve increases the blood flow through the tongue (*e g* Erici and Uvnas 1952) This increase of flow is not caused by antidromic impulses in afferent fibres, according to Folkow (1955) but mediated by efferent fibres Results obtained from studies on salivary secretion (Emmelin 1967, p 595) demonstrated a nervous control of the salivary glands also It is therefore probable that the chorda tympani nerve mediates nerve impulses not only to the central nervous system but also from it, though no recording of such impulses apparently has been published (Emmelin 1967 p 599) The present investigation was made with the aim of recording efferent impulses in the chorda tympani nerve

Methods

Adult Sprague Dawley rats were used They were anesthetized with Hypnorm® (Leo) Hypnorm® is a combination of a narcotic analgesic, 0 2 mg/ml of fenthanyl and a tranquillizer 10 mg/ml of fluanison especially adapted for veterinary use The initial dose of 0 5 ml/kg b wt was injected i m The analgesic was in most cases repeated after 30 min with the same dose and then when necessary with additional smaller doses Barbiturates were avoided for reasons that will be discussed later Gallamine iodide was often administered as a muscular relaxant when [illegible] free The [illegible] it divide [illegible] of record [illegible] systemic blood pressure was recorded in one femoral artery A polythene tube was inserted into one femoral vein for i v injections The rectal temperature was [illegible]

Results

General characteristics of the efferent chorda tympani nerve activity

Fig 1 is intended to give an idea of the activity in the chorda tympani nerve The upper trace of Fig 1 is a record from the central part of the cut nerve The lower trace shows the simultaneously recorded activity from the tongue in the peripheral part of the nerve The two records were obtained during a flow of water followed by a flow of 0 3 M NaCl over the tongue This stimulus elicited a strong activity in the peripheral part of the nerve, as shown in Fig 1

Before any conclusions from this or other records are drawn it must be stated that the nerve impulses observable depend on the signal-to noise ratio This is an admitted limitation of all conclusions in what follows

However Fig 1 indicates that a larger number of nerve impulses came from the tongue even during "steady state", than went to it This was a general observation The efferent nerve activity ranged from 10 to about 120 imp/s Occasionally 150 imp/s was observed The mean frequency was about 50 imp/s It was in most cases too small to be summated in a manner similar to that applied to the afferent activity The ratio of small impulses to large was also less in the efferent activity than in the afferent Fig 1 shows that the efferent activity was irregular The impulses appeared more or less in bursts This is perhaps better demonstrated in Fig 2

The effect of anesthesia

Fig 2 shows the simultaneously recorded activity in the central part of the chorda tympani nerve and the systemic blood pressure before and after an i v injection of pentobarbital The rat from which the records were obtained had been anesthetized with 0 1 mg fenthanyl and 5 mg fluanison/kg b wt 2 h earlier Another dose of 0 0- mg fenthanyl and 2 mg fluanison/kg b wt was injected 30 min later The left hand

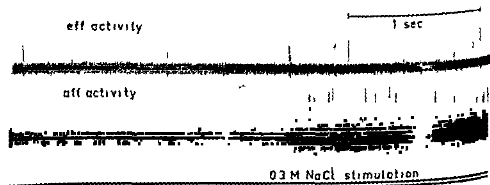


Fig 1 The upper trace displays the activity in the central part of the cut chorda tympani nerve The lower one shows the nerve activity from the tongue during a rinse of water over the tongue followed by a flow of 0 3 M NaCl with the same temperature (33° C)

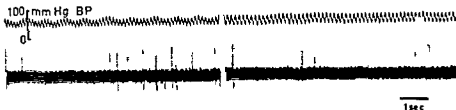


Fig 2 It shows the blood pressure and the nerve activity from the central part of the cut chorda tympani nerve immediately before and after i v injection with pentobarbital (12 mg/kg b wt) The injection lasted for 7 s The record shows a considerable decrease of the nerve activity without any decrease of the blood pressure after this injection

record of Fig 2 was then obtained 1 1/2 h after this last dose Calculation showed that the average frequency of the efferent impulses was 30 4 imp/s, SD 5 4 Immediately after the record was obtained 12 mg pentobarbital/kg b wt was injected i v over a period of 7 s Then the right hand record of Fig 2 was obtained It shows that the efferent activity became considerably depressed after the injection The average frequency decreased to 16 2 imp/s, SD 3 5 Statistical analysis showed that the difference between efferent activity before and after the injection was significant ($p < 0 001$) It can be mentioned that the pentobarbital injection decreased the efferent activity even further Thus it was about 13 imp/s after 1 min 12 after 2 min and then stabilized around 11 after 4 min

Fig 2 also shows that the injected dose of pentobarbital did not lower the systemic blood pressure On the contrary, 4 min later the blood pressure had reached a level of about 90 mm Hg This is not shown here These observations suggest that the diminution of the efferent chorda tympani activity shown in Fig 2 was not a secondary effect of circulatory failure It must be ascribed to a direct effect of the pentobarbital on the CNS

Other expts, which are not illustrated here gave similar results Thus *e g* in another animal the average frequency of the efferent chorda tympani nerve impulses was 71 2/s SD 6 4 This rat was then given 24 mg pentobarbital/kg b wt i v Ten sec after the injection the average efferent impulse frequency had decreased to 17 7 s SD 3 2 without any depression of the systemic blood pressure With larger doses of pentobarbital *it was possible to abolish completely the efferent nerve activity without* lowering the blood pressure appreciably

Thus it was a general experience that the number of efferent imp/s in the chorda tympani nerve of the rat was clearly related to the dose of barbiturate No activity at all was detected in rats anesthetized with pentobarbital alone

The later course of the efferent impulses

The lingual nerve gives off branches after it has joined the chorda tympani nerve and before its splits up close to the lingual surface of the *Corpus mandibulae* The

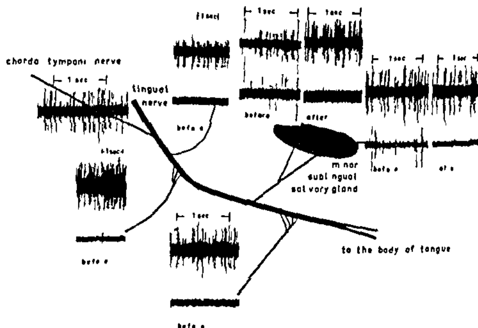


Fig 3 The results of a number of simultaneous recordings of the efferent activity are shown in this figure The upper record at each chorda lingual branch displays the activity in the uncut chorda tympani nerve while the lower one shows the activity in the branch These branches were given off on the lingual side of the Corpus mandibulae before the nerve entered the tongue The records marked before were obtained before and those marked after were obtained after the lingual nerve was cut central to its junction with the chorda tympani nerve The results suggest that most of the efferent chorda tympani nerve activity shown was mediated to structures inside the tongue

submaxillary and sublingual glands are supplied by such branches (Greene 1968 A sublingual ganglion can be assumed on comparative evidence (Mitchell 1953 p 186) However no detailed description of the branches to this ganglion their course or the ganglion itself has been found by the present author Therefore the anatomy of the region remains vague This is a source of error Despite this attempts were made to estimate the proportion of the total efferent chorda tympani activity that may be given off to structures outside the tongue Fig 3 summarizes such an attempt Before the recordings of Fig 3 were obtained the state of the chorda tympani fibres was tested by taste stimulation of the tongue Then the surface of the tongue was anesthetized with Xylocaine® until no response to taste or mechanical stimulation was observed Recordings were then obtained simultaneously from the uncut chorda tympani nerve the upper of each pair of records and from one of the branches shown in Fig 3 the lower of each pair The records marked before were obtained before and those marked after were obtained after the lingual nerve was cut central to its junction with the chorda tympani nerve After the records of Fig 3 were obtained, the tongue was rinsed with water interspersed with salt solutions until the taste response reappeared This last served as a control that no nerve block

had occurred The chorda lingual nerve was then cut close to the *Corpus mandibulae* but attempts to record from the nerve yielded no results The small number of efferent nerve impulses were probably lost in the bulk of the chorda lingual fibres Recordings from fine strands of the nerve were possible but gave no information on the quantitative relations

However, the results of Fig 3 suggest that the majority of the efferent impulses in the chorda tympani nerve were mediated to structures inside the tongue The activity recorded from the branches of the chorda lingual nerve emanated mostly from the lingual portion

In summary the results of this study demonstrate efferent activity in the chorda tympani nerve of the rat Further they show that this nerve activity is dependent upon the anesthesia used Finally they suggest that most of these nerve impulses go to the tongue

Discussion

The discussion will focus around 4 questions which arise when activity not previously observed is recorded in a nerve These are (1) what kind of nerve fibres mediated the activity observed (2) from which part of the CNS did it come (3) what structure did it supply and (4) what function did it serve?

1) Earlier anatomical studies have shown that the chorda tympani nerve contains efferent fibres In the cat and the dog about 40 % of the total number are efferent according to Foley (1945) In the rat the exact figure is unknown but there is no reason to expect a different ratio from that found in the dog and the cat Since there are in all about 1000 fibres in the chorda tympani nerve of a rat (Beidler 1969) this suggests that about 400 of them are efferent These fibres are autonomic according to Foley (1945) The rate of nerve impulses in such fibres is in general less than or around 1/s (*cf* Hillarp 1960 p 989) The impulse frequency which can be expected from such a population of nerve fibres thus agrees well with the frequency observed in the present study None or very few of these fibres are sympathetic (Foley 1945) Therefor, it is likely that the activity described here emanated from the preganglionic parasympathetic fibres of the chorda tympani nerve

2) The observation that these impulses disappeared when barbiturates were administered suggests that they originated from parts of the CNS which are more depressed by barbiturates than by morphine like substances It is known (Ingvar and Nilsson 1961) that a neuroleptic analgesic affects the cortex much less than barbiturates do It has also been reported in man that the patient possesses some ability to communicate with his surrounding even at a surgical level of neuroleptic analgesia (Ingvar and Nilsson 1961) This indicates that the cortical centres may remain relatively active However the effects of these substances on other parts of the CNS seem to be little known (Goodman and Gilman 1968) Therefore it seems that the difference in this respect observed here between barbiturates and neuroleptic analgesics cannot be used in the attempts to find the origin of these impulses but it

may be worthwhile to follow these impulses in an antidromic direction, into the CNS

3) It is well known that fibres of the chorda tympani nerve supply the salivary glands (*cf* Emmelin 1967) It seems further that there is a direct vascular innervation by efferent chorda tympani nerve fibres (Gautvik 1970) Efferent fibres of the chorda tympani nerve may also affect the sensitivity of the taste receptor cells (*cf* Halpern 1967, p 213)

With regard to the salivary innervation, it seems likely that some of these impulses were intended for salivary structures, especially the sublingual and the submaxillary glands However, the results of Fig 3 indicate that the majority of the impulses observed was mediated to structures inside the tongue This excludes the glands as the main targets, because they are situated outside the tongue But there are some small serous glands in the posterior part of the tongue of the rat (Fish, Malone and Richter 1944) which may have been the targets for the impulses Two objections may be raised against to this First, these glands are situated in a part of the tongue which judged by the sensory response of the chorda tympani nerve, is not supplied by this nerve Second it seems improbable that these small glands would have received more of the activity observed than the large ones apparently did It may therefore be concluded that at least part of the efferent nervous activity described here was destined for some other structure than the salivary glands The two other possibilities will not be discussed here but in a later study

4) There are at least three functions which might be expected to involve activity in the efferent fibres of the chorda tympani nerve The first is the regulation of blood pressure This was studied by Celander and Folkow (1951) who obtained a negative answer The sensitivity to barbiturate also seems to exclude this The two other functions are related to food and water intake and body temperature regulation Unfortunately they are both blocked by barbiturates and also are affected by neuroleptic analgesics (Goodman and Gilman 1968) Therefore, it is impossible to choose between these two at present

In summary this discussion indicates that efferent impulses in the chorda tympani nerve can be expected on anatomical grounds The sensitivity of this activity to anesthesia indicates that it is predominantly linked to "higher" functions It is also suggested that the activity described has tasks other than controlling secretion from salivary structures

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The Intracellular pH′ in the Brain in Acute and Sustained Hypercapnia

By

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Abstract

MESSETER, K and B K SIESJO *The intracellular pH′ in the brain in acute and sustained hypercapnia* Acta physiol scand 1971 83 210—219

The regulation of intracellular pH in the brain was studied in rats exposed to about 11 % CO_2 for periods of 15 min to 72 h At the end of each exposure period cisternal cerebrospinal fluid, as well as the supratentorial parts of the brain were sampled and analysed for the CO_2 contents The intracellular HCO_3 concentration was calculated assuming extracellular volumes of 12 15 and 20 %, respectively, and the intracellular pH was derived from the HCO_3 concentration and the mean tissue CO_2 tension Acute hypercapnia (15 min) was associated with an increase in the intracellular HCO_3 concentration of about 6 meq/kg of i c water With an extracellular volume of 15 % the corresponding decrease in pH_i was from 7 06 to 6 93 When the hypercapnia was upheld there was a further increase in the intracellular HCO_3 [illegible] pH_i to about 7 03 Most if not [illegible] h An analysis of the regulating [illegible] bolic consumption of acids and [illegible] bout a third of the total accum-

It has been reported that sustained hypercapnia is accompanied by a gradual increase in the total acid labile CO_2 content of the brain (Nicholls 1958, Weyne et al 1968, cf also Siesjo 1965) This finding indicates that during the hypercapnia the bicarbonate concentration increases in either the extra- or intracellular spaces, or in both spaces, and that the corresponding pH values are gradually shifted in the direction of those measured in normocapnia However, as long as the extra- and intracellular bicarbonate concentrations are not simultaneously measured the regulation of intracellular pH′ will remain unknown

In a previous communication we described sequential changes in the extracellular bicarbonate concentrations and in the extracellular pH, in rats exposed to carbon dioxide for periods ranging from 5 min to 72 h (Messeter and Siesjo 1971 a) The present communication is concerned with the corresponding intracellular changes under the same experimental conditions The intracellular bicarbonate concentra-

tions were calculated after correction for the amount of bicarbonate contained in the blood and extracellular volumes of the tissue Since an exact figure for the brain extracellular volume is lacking, the intracellular bicarbonate concentration was calculated using extracellular volumes of either 12 %, 15 % or 20 % of the tissue weight It will be shown that irrespective of the extracellular volume assumed, the results indicate that hypercapnia is accompanied by an efficient regulation of the intracellular pH′ in the brain Preliminary reports of the present findings have been published (Messeter and Siesjö 1970, Siesjo and Messeter 1971)

Methods

Most of the present experiments were identical to those reported in a preceding communication (Messeter and Siesjo 1971 a) For this reason, only the main features of the experimental procedures will be described There were 2 main groups In one, unanesthetized rats were exposed to a gas mixture containing about 11 % CO_2 and 30 % O_2 which was flushed

intermittent heating At the end of the experiment, and after that at least two sets of blood analyses had been performed, cisternal cerebrospinal fluid was sampled by puncturing the atlantooccipital membrane, and the brain was frozen *in situ* by pouring liquid nitrogen into a funnel fitted onto the exposed skull bone

thane 11 % CO_2 and 30 % O_2 was delivered and mechanical hyperventilation was started The animals were then exposed to the gas mixture for either 15 or 45 min before the CSF and the tissue were sampled. During the hypercapnia, at least two sets of blood analyses were

pratentorial parts of the brain were , Ponten and Siesjo 1964) For each three individual analyses The water content was measured by drying pieces of tissue at 105° C overnight

The intracellular pH (pH_i) was calculated from the following equations

$$(HCO_3^-)_t \approx T_{CO_2} - P_tCO_2 \cdot S_1 \tag{1}$$

$$(HCO_3^-)_i \approx \frac{(HCO_3^-)_t - (HCO_3^-)_{CSF} \cdot V_{ECF} - (HCO_3^-)_{Bl} \cdot V_{Bl}}{V_i} \tag{2}$$

$$pH_i = 6.12 + \log \frac{(HCO_3^-)_i}{P_iCO_2 \cdot S_2} \tag{3}$$

In these equations $(HCO_3^-)_t$ and $(HCO_3^-)_i$ denote the whole tissue bicarbonate content (mmol/kg of wet tissue) and cellular water), respectively to the arterial CO_2 tensions

he intracellular water (S_2) (Siesjo 1962 b) The CSF CO_2 dissolved ($P_tco_2 \cdot S_2$)

from the total CO_2 content The mean bicarbonate content in whole blood was determined from the Siggaard Andersen alignment nomogram The blood volume of the tissue (V_{Bl}) was assumed to be 3 % of the tissue weight in all experiments The extracellular volume (V_{ECF}) was assumed to be either 12 %, 15 % or 20 % (see Discussion) and since the mean water content was 794 g/1000 g of wet tissue, the intracellular volumes (V_i) derived were 64 61 and 57 %, respectively The pK_1' for carbon acid, finally, was taken to be 6 12 (Siesjo 1962 c)

Results

In all of the experiments to be reported, the mean arterial blood pressure exceeded 120 mm Hg, the arterial oxygen tension exceeded 100 mm Hg, and the hemoglobin content was higher than 13 5 g/100 ml The administration of about 11 % CO_2 to the unanesthetized animals led to overt hyperventilation but in no case were convulsions or signs of narcosis observed

Table I gives the measured arterial CO_2 tensions and pH values, as well as the total CO_2 and tissue water contents The results confirm previous findings which have shown appreciable increases in the tissue CO_2 content in sustained hypercapnia (Nicholls 1958, Weyne *et al* 1968) and they indicate that little further change is seen after 24 h ($0 8 < p < 0 9$)

Table II demonstrates the intracellular bicarbonate concentrations and pH values obtained in the control group and in the groups exposed to carbon dioxide for 15 min, 3 h and 48 h, respectively In each group the intracellular parameters were calculated assuming extracellular volumes of 12, 15 and 20 %, respectively The

TABLE I The influence of acute and sustained respiratory acidosis on acid base parameters in arterial blood brain tissue and cisternal cerebrospinal fluid in groups of rats exposed to about 11 % CO_2 in the inspired air (means±S E) Number of experiments within parenthesis The number of CSF measurements in each group was 5—10

Time of exposure	Measured values				
	Arterial blood		Tissue		CSF
	P_{CO_2} mm Hg	pH	TCO_2 mmol/kg	H_2O % by weight	TCO_2 mmol kg
Control (14)	37 4 ±0 6	7 43 ±0 01	13 4 ±0 2	79 3 ±0 4	29 6 ±0 4
15 min (10)	84 4 ±1 1	7 13 ±0 01	18 8 ±0 2	79 4 ±0 7	33 6 ±1 2
45 min (14)	86 1 ±0 7	7 13 ±0 01	20 6 ±0 2	79 3 ±0 7	37 1 ±1 1
3 hrs (10)	84 0 ±0 6	7 20 ±0 01	22 4 ±0 2	79 3 ±0 6	38 8 ±1 2
24 hrs (8)	87 1 ±1 5	7 27 ±0 01	24 4 ±0 4	79 6 ±1 5	48 2 ±0 5
48 hrs (11)	85 0 ±1 3	7 30 ±0 01	24 3 ±0 2	79 4 ±0 5	48 3 ±0 9
72 hrs (7)	85 1 ±1 6	7 30 ±0 01	24 7 ±0 4	80 1 ±0 4	50 6 ±0 7

TABLE II The calculated brain intracellular bicarbonate concentrations and pH in groups of rats exposed to 11 % CO_2 for periods of 15 min to 48 h (means±S E) The acid base parameters were derived for a 12 %, a 15 % and a 20 % extracellular space Number of experiments within parenthesis

Time of exposure	12 % ECV		15 % ECV		20 % ECV	
	$[HCO_3^-]$ meq/kg i c water	pH_i'	$[HCO_3^-]$ meq/kg i c water	pH_i'	$[HCO_3^-]$ meq/kg i c water	pH_i'
Control (14)	12 8 ±0 3	7 09 ±0 01	12 0 ±0 3	7 06 ±0 01	10 6 ±0 3	7 00 ±0 01
15 min (10)	18 6 ±0 3	6 94 ±0 01	18 0 ±0 3	6 93 ±0 01	16 8 ±0 3	6 90 ±0 01
3 hrs (10)	23 0 ±0 3	7 04 ±0 01	22 3 ±0 3	7 03 ±0 01	21 1 ±0 4	7 00 ±0 01
48 hrs (11)	23 8 ±0 3	7 05 ±0 01	22 7 ±0 3	7 03 ±0 01	20 7 ±0 3	6 99 ±0 01

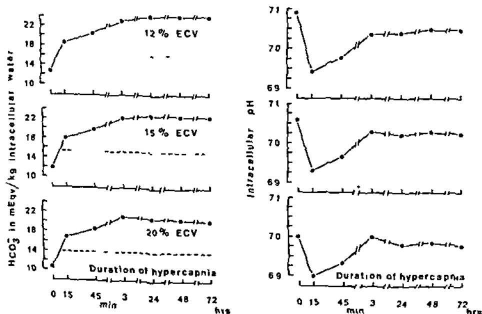


Fig 1 The intracellular bicarbonate concentrations and the intracellular pH of rats exposed to about 11 % CO_2 for periods from 15 min to 72 hrs The values were calculated for an assumed extracellular volume of 12, 15 and 20 %, respectively The stippled lines in the left panel represent the increase in the intracellular HCO_3 concentration which can be assumed to be due to pure physico-chemical buffering The increase was calculated on the assumption that the in vivo buffer capacity $\frac{\Delta \log P_{CO_2}}{\Delta pH}$ in acute hypercapnia (15 min) should be equal to that determined in vitro (see Discussion) The gradual increase in the HCO_3 concentration above the values given by these lines probably represents both metabolic consumption of acids and influx of HCO_3 from the extracellular fluid (see Discussion) Note appreciable regulation of intracellular pH in sustained hypercapnia

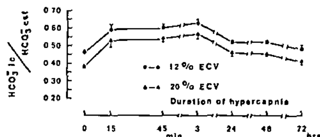


Fig 2 The ratio between the intracellular and the CSF bicarbonate concentrations in hypercapnia as a function of time The values were derived for a 12 % and a 20 % extracellular fluid space Means ± S E

table shows that although the assumption as to the size of the extracellular space will influence both the control HCO_3 concentration and the pH_i' values, as well as the magnitude of the pH shifts in acute or sustained hypercapnia, certain general conclusions seem valid Thus, the intracellular HCO_3 concentration was approximately doubled in sustained hypercapnia Furthermore, a substantial part of the bicarbonate accumulation occurred after the acute phase of hypercapnia (15 min) Finally, the intracellular pH′ seems very efficiently regulated since in the chronic state the intracellular pH′ did not seem to deviate from the normal pH′ by more than maximally 0 04 units The significance of this figure may be more obvious if it is recalled that whole blood *in vitro* will decrease in pH by about 0 2 units for the same increase in P_{CO_2}

Fig 1 illustrates the changes in the intracellular HCO_3 concentrations and pH values as functions of time The figure demonstrates the accumulation of bicarbonate in the intracellular space which can be assumed to be due to other mechanisms than re physico chemical buffering (increases above broken horizontal lines, see discus-) Irrespective of the extracellular fluid volume assumed, very little further ccumulation of bicarbonate seemed to occur after the first 3 h Accordingly, the regulation of pH_i' appeared close to maximal at this time This conclusion was supported by a statistical analysis which failed to show any significant differences in the HCO_3 concentrations or pH_i' values between the 3 h and 72 h groups, irrespective of the extracellular volume assumed ($p > 0\ 05$)

Fig 2 shows the ratio between the intra- and extracellular bicarbonate concentrations, derived for a 12 and a 20 % extracellular volume There were significant increases in the ratio in acute hypercapnia (15 min) as well as in the 45 min and the 3 h groups ($p < 0\ 001$), followed by a gradual decrease towards the control values The figure demonstrates that the rapid accumulation of bicarbonate in the cells during the first 3 h occurred in spite of an increased chemical diffusion gradient in the reverse direction

Discussion

The present results have demonstrated that sustained hypercapnia with an increase in the tissue CO_2 tension from about 45 to about 90 mm Hg is accompanied by a marked accumulation of bicarbonate in the intracellular space of the brain This

accumulation, virtually all of which occurs during the first three hours of hypercapnia, is sufficient to bring pH$'_i$ back to values which are less than 0.05 units below the pH$_i'$ of the normocapnic control animals. This degree of regulation is far in excess of what can be expected from pure physico-chemical buffering and we will discuss the possible mechanisms involved. However, it seems appropriate to start the discussion by bringing up a few conceptual and methodological problems.

Concepts and assumptions. In any study of the intracellular pH' in tissues like the brain two main difficulties are encountered. One is the conceptual difficulty of defining pH in a system which contains different types of cells, and multiple compartments within each cell (Caldwell 1956, Waddell and Bates 1969). In view of the complexity of the system it would seem advisable to drop the pH term altogether (Siesjö and Pontén 1966), but since it appears to be the convention to retain the term we have chosen to call it an equivalent intracellular pH (pH$_i'$). The pH values derived should then be interpreted as the pH existing in a homogenous compartment which has the same bicarbonate concentration and the same CO_2 tension as the corresponding *mean* values for the intracellular space. The use of the term should not be allowed to obscure the fact that there may be differences between *e.g.* nerve and glial cells, and between *e.g.* cytoplasm and mitochondria in each of these cell types. These possible differences should be born in mind when extra- and intracellular bicarbonate concentrations, or the corresponding pH values, are compared.

The second main difficulty in studies of the intracellular pH' in a tissue like the brain is methodological and concerns the assessment of a tissue CO_2 tension as well as proper corrections for the bicarbonate contained in the blood and the extracellular volumes of the tissue. The CO_2 tension may present the least problem since it appears possible to define a mean tissue CO_2 tension in the tissue, and also to measure it, using CSF as a tonometer (Pontén and Siesjö 1966, Siesjö *et al.* 1967, Brzezinski *et al.* 1967). Repeated control measurements have also shown that in normotensive animals the mean tissue CO_2 tension can be calculated from the arterial CO_2 tension with sufficient accuracy.

The correction applied for the blood bicarbonate content is a small one and it is of little importance if the actual blood volume should increase by *e.g.* one per cent during the hypercapnia. The extracellular HCO_3^- concentration is more critical in the calculations. Presently there seems to be an almost general agreement that the ECF volume in the brain falls within the range of 10 to 20 %. In the original report of Rall *et al.* (1962) who used ventriculocisternal perfusion with inulin the figure given was about 12 %. In a later report (Rall and Fenstermacher 1971) values of 17.0 and 14.5 % has been given for dog cerebral cortex and periventricular white matter, respectively (see also Oldendorf and Davson 1967). In rats Woodward *et al.* (1967) reported an extracellular volume of 13—15 %. Although the extreme figures used in the present calculations may be useful for illustrating the dependence of the calculated figures upon the assumed extracellular volume experimental results thus seem to support a value of about 15 %. We will therefore use this figure in the following.

In the calculation of the intracellular acid base parameters the assumption is made that the bicarbonate concentration in the CSF is representative of the extra cellular bicarbonate concentration This assumption is probably well justified in all situations which approach that of a steady state (see Fencl *et al* 1966) However in the most acute hypercapnic situations the CSF analyses probably underestimate the true extracellular bicarbonate concentration Thus, although the effect on the calculated intracellular bicarbonate concentration is small even if the increase in the CSF bicarbonate is only 50 % of that in the extracellular fluid proper, it should be recalled that the decrease in pH_i' will be slightly underestimated, at least in the 15 min group, and possibly also in the 45 min group

Factors regulating intracellular pH' It has recently been pointed out that there are at least 3 mechanisms which will contribute to the pH regulation in hypercapnia physico chemical buffering, consumption of organic acids and trans-membrane fluxes of H^+ or HCO_3 (Siesjo and Messeter 1971, see also Brown 1971 Waddell 1971) We will discuss how these mechanisms may have contributed in regulating pH_i' in the present experiments The discussion may be facilitated if we give numerical values for the degree of pH regulation It has been the convention to express this by calculating a "buffer capacity", commonly as the $\Delta \log P_{CO_2}/\Delta pH$ or the $\Delta[HCO_3^-]/\Delta pH$ ratios (see Siesjo and Sorensen 1971, Woodbury 1965) It should be recalled that the figures obtained do not agree with the buffer capacity expression in a physicochemical sense but that they should be considered as figures which express the total 'buffering" irrespective of its mechanisms ('physiological buffering , see Brown 1971, Siesjo and Sorensen 1971) The numerical values obtained in the present experiments are given in Table III The significance of the figures is perhaps more obvious if we recall that whole blood *in vitro* gives a $\Delta \log P_{CO_2}/\Delta pH$ value of about 1 6 and a $\Delta[HCO_3^-]/\Delta pH$ value of about 30

The *physico chemical buffering capacity,* which expresses the efficiency of the fixed buffer groups in limiting the pH change, is difficult to measure in tissues H

TABLE III The estimated changes in the apparent intracellular buffer capacity during acute and sustained hypercapnia expressed as $\frac{\Delta \log/P_{CO_2}}{\Delta pH}$ and $\frac{\Delta [HCO_3^-]}{\Delta pH}$ The values were calculated from the data given in Table I, and Fig 1, derived for a 15 % extracellular volume

Time of exposure	15 % ECV	
	$\frac{\Delta \log P_{CO_2}}{\Delta pH}$	$\frac{\Delta [HCO_3^-]}{\Delta pH}$
15 min (10)	2 3	46
45 min (14)	3 3	88
3 h (10)	10 1	343
24 h (8)	7 7	273
48 h (11)	10 5	357
72 h (7)	9 0	265

ever, titrations on brain homogenates indicate that it is about 1.5—1.7 when calculated as $\Delta \log P_{CO_2}/\Delta pH$ (Siesjö and Messeter 1971). This is to say that if physico-chemical buffering was the only mechanism regulating pH_i' we would expect that an increase in the tissue CO_2 tension from 45 to 90 mm Hg should decrease pH_i' from 7.06 to about 6.87, and that the intracellular HCO_3^- concentration should increase from 12 to about 15 meq/kg of H_2O.

Consumption of organic acids. The present experiments have demonstrated that acute hypercapnia (15 min) gives a 'buffer capacity' which is significantly higher than that expected from pure physico-chemical buffering (see also Roos 1965, Kjällquist *et al.* 1969, Granholm and Pontén 1969). This conclusion is unaffected by the assumption as to the size of the extracellular space (12 to 20 %) as well as by an underestimation of the extracellular bicarbonate concentration by 50 %. Since 15 min of hypercapnia seems too short a period to allow appreciable transmembrane fluxes of H^+ or HCO_3^-, it appears probable that consumption of organic acids plays a role. We have previously assumed that the decrease in the phosphocreatine content and oxidation of part of the pools of lactate, pyruvate and α-ketoglutarate provided the necessary basic equivalents to explain the additional 'buffering" (Siesjö and Messeter 1971). We have later found that also the glutamate content of the tissue decreases in hypercapnia (Siesjö *et al.* 1971, Messeter and Siesjö 1971 b) and if we make the assumption that a decrease in the intracellular glutamate leads to a mole to mole increase in buffer base we find that 15 min of hypercapnia is associated with an increase in buffer base of 2.5—3 meq/kg of i.c. water. This is approximately sufficient to explain the increase in the intracellular HCO_3^- concentration above that expected from physico-chemical buffering and to increase the $\Delta \log P_{CO_2}/\Delta pH$ value from 1.5—1.7 to about 2.3. Since there is a further fall in the glutamate content when the hypercapnia is prolonged from 15 to 45 min it is possible that part of the bicarbonate accumulation also during this period is caused by consumption of acid.

Transmembrane fluxes of H^+ or HCO_3^-. After 45 min of hypercapnia there are no further changes in the tissue contents of phosphates or organic acids (Messeter and Siesjö 1971 b). We may thus assume that the further accumulation of HCO_3^- is caused by transmembrane fluxes of H^+ or HCO_3^-. This further accumulation of HCO_3^- was only about 3 meq/kg of i.c. water or about 30 % of the total accumulation in sustained hypercapnia. However, if we take into account that an accumulation by ion fluxes occurred also during the first 45 min we may conclude that maximally 40 % of the total HCO_3^- accumulation in sustained hypercapnia could be due to transmembrane fluxes. However, we can draw this conclusion only by tacitly assuming that there are no other acid-consuming metabolic mechanisms than those considered here. If such mechanisms exist the assumed role of the fluxes may have to be reevaluated.

We have previously discussed if the transmembrane fluxes of H^+ or HCO_3^- in sustained hypercapnia are 'passive' or "active' and a tentative analysis of the transport work indicated that most of the HCO_3^- accumulated by flux from the extracellular fluid must be the result of an active transport (Siesjö

1971) The present results have demonstrated that the period of intracellular accumulation of bicarbonate is accompanied by an increased intracellular/extracellular bicarbonate ratio It would seem as if such a change in the HCO_3 gradient should favour outflux of HCO_3 from the cells and thus support the conclusion that any flux of HCO_3 into the cell must occur at the expense of metabolic energy

We may summarize by tentatively stating that out of the total increase in the intracellular HCO_3 concentration of 11 meq/kg, about 3 meq/kg should be due to physico chemical buffering while approximately 4 meq/kg each can be ascribed to metabolic consumption of acids and to transmembrane ion fluxes, respectively This is to say that if only physico-chemical buffering had existed the pH_i' should fall to and remain at, about 6 87 The consumption of acids is then responsible for a return of pH to about 6 97 while transmembrane flux of H^+ or HCO_3 brings the pH_i' back to about 7 03

Relation to previous work The total accumulation of HCO_3 in the brain during the prolonged hypercapnia is in good agreement with the results reported by Nicholls (1958) and by Weyne *et al* (1968 1970, 1971) In the experiments by Weyne *et al* the P_{CO_2} was brought back to normal after a sustained period of hypercapnia and the authors found a much higher bicarbonate content than in normocapnic animals Similar findings have been reported by Kazemi *et al* (1967), who studied the *in vitro* dissociation curves of the brain after prolonged hypercapnia The results obtained by the different groups seem to demonstrate that a large part of the bicarbonate which has accumulated during sustained hypercapnia is unrelated to physico-chemical buffering but in the absence of information on metabolic changes and on changes in the extracellular HCO_3 concentration quantitative conclusions nnot be drawn At any rate, the results obtained in the present as well as in the ious studies seem to leave no doubt that the brain cells posess efficient echanisms for regulating the intracellular pH in hypercapnia, as do other cells in the body (see Brown 1971)

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Characterization of Simple and Composite Uptake Systems in Cells and Tissues by Competition Experiments

By

ERNST H BÁRÁNY

Received 19 March 1971

Abstract

BÁRÁNY, E H *Characterization of simple and composite uptake systems in cells and tissues by competition experiments* Acta physiol scand 1971 *83* 220—234

The first step of active uptake of a substance is taken to be the reversible combination with a primary binding site (receptor) by a first order reaction Such a binding is characterized by an affinity constant Several kinds of receptors can be simultaneously present in a cell or tissue one then has a

their ability competitively to depress the uptake of the test substance For these substances only t e concentrations in the medium are required The mathematics of such competition experiments are derived for a simple and for a composite system consisting of two independent systems with completely overlapping but not identical affinity spectra A formula is derived which allows one to est n a e the affinities of any substance to the two receptor systems by measurements at four concentrations of the substance In the case of incompletely overlapping affinity spectra, the systems can e her be studied separately or as if there were complete overlap

There are a number of wide-specificity transport systems in the body Examples are the organic acid secreting systems in the proximal tubules of the kidney, the eye and the choroid plexus Similar but not identical systems exist in the liver and very probably in other places There are base transporting systems of similarly wide specificity These systems often are studied *in vitro* where the tissue is allowed to accumulate the transported substance

If one wants to study the range of specificity of a wide-specificity system, it is not practical to perform direct uptake experiments with the hundreds of substances which might have some affinity to the system This would require an analytical method for each of the substances Instead competition experiments are usually performed where the ability of substances to interfere with the transport of a test substance are studied One then only has to analyze the test substance concentration in the tissue and the medium

As Christensen (1966, see also Inui and Christensen 1966) has pointed out, one should always suspect that a transport system is composite until the opposite is proved For evident reasons, this warning is especially relevant for wide specificity systems It is therefore necessary to understand the competition experiment in a tissue with several uptake systems for the substance under study, or, shorter, a composite uptake system

This was the reason for the work reported in the present paper, which was started because of difficulties encountered in understanding results obtained in uptake experiments *in vitro* where the organic acid iodipamide was incubated with slices of kidney cortex and liver, ciliary processes and choroid plexus from several species Iodipamide turned out to be taken up by several systems (Bárány, unpublished)

It should be mentioned at the start that we deal with specific, active transport processes, not diffusion into the extracellular space or throughout the water phase of the tissue Thus, when "concentration in the tissue' or "tissue/medium ratio ' is mentioned, the terms refer to that part of the tissue concentration which remains when the passively accumulated portion has been deducted This "passive ' portions would for instance be found by an incubation at 0° C or in total absence of energy supply or after total inhibition of the transport system by a competitor

The treatment is a simple extension of well known Michaelis—Menten mathematics Specific uptake of one single substance by a (presumably) single uptake system has repeatedly been shown to follow such kinetics, at least approximately (see Weiner 1967 for references) There is an evident analogy between the problem dealt with here and the work done by others on drug receptor interaction This analogy is not only formal One especially useful concept introduced by Ariens, that of ' intrinsic activity ' has proved its worth also in the present context To emphasize the analogy, some of the notations used by Ariens (1964) have been used here too

In the first part of the following, the mathematics of simultaneous uptake of many substances by a simple and by composite uptake systems are formulated This part lays the ground for the second part, where the mathematics of the competition experiments are derived for a simple system and for an uptake system with two components A formula is derived which allows one to estimate the affinities of a substance to both components of a 2 component system from results of a competition experiment The practical approach to an unknown tissue and the limitations due to imprecise data are finally discussed

I Uptake

A *The simple uptake system One kind of primary binding site many substances*

1 *The free receptor fraction F The affinity spectrum*

The first and reversible step of *active* uptake is the association with ' the primary binding site ' For shortness and in analogy with the nomenclature of pharmacology, the primary binding site will be called the receptor It is assumed that all the recep-

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of the receptor proper but is determined by further steps of the uptake process, for instance ease of unloading (inside the cell) of the transported material from a carrier (cf Forster 1967) or ease of back diffusion Anyhow, α is specific for any combination between a specific substance and the receptor and the uptake system therefore has an α *spectrum* besides the affinity spectrum

Thus, one has, using (1)

$$\begin{aligned} V_i &= \alpha_i[RS_i] \\ &= \alpha_i F[r]\,[S_i]/k_i \end{aligned} \qquad (3)$$

We shall now consider what happens if one of the substances is present in high concentration

It follows from (2) and (3) that as any

$$\begin{aligned} [S_i] &\to \infty \\ F[S_i]/k_i &\to 1 \\ V_i &\to \alpha_i[r] \end{aligned}$$

thus the maximum velocity of uptake is

$$V_{\max_i} = \alpha_i[r] \qquad (4)$$

This is understandable since in the extreme case the substance present in high concentration monopolizes the receptors

Combining (3) and (4) one finds

$$V_i = V_{\max_i} F[S_i]/k_i$$

which can easily be transformed to the conventional Michaelis—Menten formula for reaction velocity — which also often is applied to uptake

In the above, *α S, V_{max} and k are characteristic for each substance and its interaction* with the uptake system and thus have individual subscripts The value $[r]$ depends only on the tissue and F on the affinity spectrum of the receptor and *all* the competing substances present F and $[r]$ are the same for all the substances

3 *Tissue/medium ratios*

We assume that the conditions are such that the specifically accumulated tissue concentration of all the substances dealt with is proportional to the uptake velocity V and to the duration of the incubation t This will always be true in short enough experiments Then, using equations (3) and (4) the tissue/medium or slice/medium concentration ratio Q for any substance S_i belonging to the system becomes

$$Q_i = tV_i/[S_i] = tF[r]\alpha_i/k_i = tF\,V_{\max_i}/k_i \qquad (5)$$

Here, V_{max}, α and k belong to the individual substance while F and $[r]$ are common With increasing concentration S_i the free fraction F decreases and at infinite concentration, which gives V_{max} Q_i is in fact zero (self depression of the tissue/medium ratio)

With vanishing chemical concentrations of *all* transported substances, the value of Γ approaches 1 0 The corresponding Q are the theoretical maximum,

$$Q_{\max_i} = t\alpha_i[r]/k_i = tV_{\max_i}/k_i \tag{6}$$

Combining (5) and (6) one obtains

$$Q_i = \Gamma Q_{\max_i} \tag{7}$$

Since with tracer amounts of labelled substances it often is easy to measure $Q_{\max}$ this is a useful as well as illuminating relation

Since the free receptor fraction Γ is common to all substances *with affinity to the* receptor, equation (5) and (7) show that in a one-receptor uptake system any change in Γ affects the Q value of all present substances equally Any change in a concentra*tion [S] or an affinity 1/k which changes F causes percentually identical changes in* the tissue/medium ratios Q of *all* the substances competing for the system

Conversely, if the addition of one or the other competitor changes Q differently for different substances, this is a strong reason to suspect that the substances are taken up by at least two different systems, the Γ of which are differently affected by the addition of the competitor

Admittedly one can imagine situations where addition of a competitor causes a change in α, the specific uptake velocity, for some other substance, but such inter actions beyond the receptor level must be a special case

B *The composite uptake system*

1 *Several receptors, many substances*

We make the same simple assumptions as for accumulation by a single uptake system. Accumulation by several independent systems A, B in a tissue thus becomes the sum of independent components By extension of (7) the tissue/medium ratio becomes

$$Q_{A \ldots B} = \Gamma_A Q_{\max_A} + \Gamma_B Q_{\max_B} + \tag{8}$$

For each substance which is accumulated by systems A, B, an expression (8) is valid

It is immediately apparent why in a composite system different substances can be depressed unequally much by the addition of a competitor which in general will affect the different Γ values differently

2 *Selective depression of one component in a composite uptake system*

If a composite uptake system is to be analyzed, it may be desirable to depress one or more components selectively A common competitor in the medium, suitably selected may depress one component more than the others, but in case of total overlap (see below) only at the cost of depression of all other components It must be remembered also, that a competitor, which, say, depresses B more than A, can virtually abol h

the contribution of B to the uptake of one substance (with $Q_{max_A} \gg Q_{max_B}$) while for some other substance (with $Q_{max_B} \gg Q_{max_A}$) B still may contribute considerably or even dominate the total uptake

The highest possible selectivity is attained if the most selective substance is used *in sufficient concentration* This may be impossible because of limited solubility, toxicity or other reasons Can selectivity then be increased by adding several competing substances of less selectivity? This depends on the highest $[S]/k$ of the most selective substance that can be used and on the degree of selectivity of the others that may be used

The discrimination ratio F_B/F_A shows how much more of one system is unsaturated than of the other when $F_B/F_A > 1$, system B is less depressed than system A

Let S_1 be the most selective competitor available and $[S_1]$ its concentration Then, from (2)

$$\left(\frac{F_B}{F_A}\right)_1 = \frac{1+[S_1]/k_{1_A}}{1+[S_1]/k_{1_B}}$$

What is required of a substance S_x with less selectivity than S_1 for it to increase the discrimination ratio further? In its presence

$$\left(\frac{F_B}{F_A}\right)_{1+x} = \frac{1+[S_1]/k_{1_A}+[S_x]/k_{x_A}}{1+[S_1]/k_{1_B}+[S_x]/k_{x_B}}$$

If the addition of the S_x-terms to the numerator and the denominator is to increase the discrimination ratio, the following is required

$$\frac{k_{x_B}}{k_{x_A}} > \left(\frac{F_B}{F_A}\right)_1$$

Since the discrimination ratio reaches k_{1_B}/k_{1_A} only when $[S_1] = \infty$ one has as a rule

$$\left(\frac{F_B}{F_A}\right)_1 < \frac{k_{1_B}}{k_{1_A}}$$

and it can easily happen that the selectivity of depression of the one system can be increased by the addition to the medium of several somewhat less specific competitors besides the most specific one The selectivity cannot, however, exceed that which would have been achieved with the most selective substance in high concentration

II Characterization of uptake systems by means of competitors

A *Competition in a simple uptake system*

1 *Arithmetic solution*

It is a common procedure to study an uptake system by means of a test substance S_T which is allowed to accumulate and a variety of analogs or otherwise inte[illegible] compounds S_I which act as inhibi[illegible]

$1/k_I$ for the inhibitors result from the experiments Thus, the affinity spectrum of the receptor can be obtained without analytical methods for all the inhibitors It is evident, however, that such competition data give no information about intrinsic velocities of uptake α for the different competitors nor can they tell anything about the concentration $[r]$ of receptor in the tissue But α may in fact be less a property of the receptor than of further steps in the uptake system and $[r]$ is a property of the tissue not the receptor Thus, competition experiments do give the essential information about the receptor

With a test substance S_T that can be used in tracer amounts, (=chemically negligible concentration) a true Q_{max_T} is available and one can calculate k_I for any inhibitor S_I from the equation

$$k_I = [S_I] \quad \frac{Q_T}{Q_{max_T} - Q_T} \qquad (8)$$

which is easily derived from (2) and (7) Here Q_{max_T} and Q_T are the tissue/medium ratios of the test substance in the absence and the presence of the inhibitor S_I respectively Only one concentration $[S_I]$ is necessary if one does not need to check the truth of the assumption that S_I is a competitive inhibitor

If the inhibitor S_I is not the only receptor-occupying substance present in appreciable amounts, for instance if the test substance S_T has to be present in appreciable concentration in the medium for measurable amounts to accumulate in the tissue, the formulae become slightly more complex

Let the sum of all $[S]/k$ except $[S_I]/k_I$ be P and let Q_{o_T} be the accumulation ratio of the test substance when $[S_I] = 0$ Then one has from equation (7)

$$Q_{o_T} = \frac{Q_{max_T}}{1+P} \qquad (9)$$

$$Q_T = \frac{Q_{max_T}}{1+P+[S_I]/k_I} \qquad (10)$$

by division and rearrangement

$$k_I = [S_I] \quad \frac{1}{1+P} \quad \frac{Q_T}{Q_{o_T} - Q_T} \qquad (11)$$

Thus, if one is content with the *relative* magnitude of k_I in a series of compounds S_I is suffices to keep P constant, to measure Q_{o_T} and then only one Q_T at one $[S_I]$ for each compound If one wants absolute values of k_I one has to estimate P in addition. This can be done in various ways depending on what P consists of If it consists only of the $[S]/k$ of an extra competitor this can be left out, a Q_{max_T} obtained and equation 9 applied If P is due to the test substance S_T a Lineweaver-Burk plot of $\frac{1}{Q_T}$ against $[S_T]$ (in the absence of S_I) leads to Q_{max_T} Equation 9 then gives P

The value of Q_T to aim at in order to reduce the random errors of the estimate of k_I depends on the errors in Q_{max_T} and Q_T In uptake experiments, it is reasonable to

expect that the relative error increases as Q_T goes down, which gives approximately equal absolute errors in Q_{max_T} and Q_T. With such a constant error, the random error in k_I is minimized close to $Q_T = 1/2\ Q_{0_T}$.

2 *Graphical solution*

If one prefers a graphical solution, which allows a rough test of the assumption that S_I is a competitor, one inverts equation (10) and plots $1/Q_T$ against S_I.

$$\frac{1}{Q_T} = \frac{1+P}{Q_{max_T}} + \frac{[S_I]}{k_I Q_{max_T}}$$

Thus the y intercept is $(1+P)/Q_{max_T}$ (and should be the same with all S_I) and the slope $1/k_I Q_{max_T}$.

If one is content with relative values of k_I one needs no Q_{max_T}. Else one has to set up a special experiment for it, as mentioned in the preceding section. This, of course, is not necessary, if there is no P, because then the y intercept gives $1/Q_{max_T}$.

Dowd and Riggs (1965) have stressed the disadvantage of a plot where the inverse value of a variable with large random errors appears. The low Q_T-values have the highest relative experimental error but the largest influence on the slope and the k_I value derived from it. This criticism of the inverse plot is valid also here. Evidently, one should design the experiment in such a manner that Q_T is reasonably high at all concentrations or at least only utilize that part of the data where Q_T is reasonably certain.

B *Competition in a composite uptake system*

1 *Different kinds of overlap*

We assume that the composite nature of the system has been established either by one of the stratagems discussed by Christensen (1966) or by the method suggested in the third paragraph following equation (7) of the present paper.

Our aim is to measure the affinity spectra of the components individually, simultaneously or in separate experiments. The treatment will be limited to two-component systems. For more complex systems the reasoning is similar but the mathematics become unwieldy.

Three situations can be distinguished in the two-component case (Fig. 1).

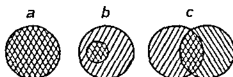


Fig. 1

a As far as is known, there is complete overlap This does not imply that overl is *in fact* complete but only that, at the present stage, we have no substance which h affinity to the one but not to the other system

b. One system overlaps the other completely but not vice versa Thus, there a *known* compounds with affinity to the "outer" system only but no *known* compou with affinity to the "inner" system only

c There is incomplete overlap and there are substances known with selective affi ity for each of the two components

In this last case, *c*, one can suppress the one system by occupying its receptors wi appropriate substances and then study the affinity spectrum of the remaining syste But one can also select a system-specific test substance or even combine both metho

In case *b*, the "outer" system can be studied by means of a specific test substan without affinity to the inner system or it can be suppressed by one or more speci depressors and the remaining "inner" system studied alone

Thus, as a rule, both *b* and *c* can be converted to virtual single uptake systems ar studied as suggested in the preceding section If this is impossible for any reaso they can be treated as case *a*

Only case *a* thus remains and needs more exhaustive treatment

2 *Two completely overlapping systems*

Call the systems *A* and *B* We assume that we do not know anything about the prop erties of systems *A* and *B* separately We are going to present formulae which allo the estimation of the properties of the components from measurements on the compo site system

In order to determine the affinity of a specific compound for the two systems, w need one test substance S_T which can easily be determined in the tissue sample W also may need a depressor S_D, a substance added in order to reduce the uptake o the test substance by the one system more than by the other Finally we need sever concentrations of the substance the affinity of which we want to study This substanc will be called the inhibitor S_I The same compound can act in all three capacities bu we shall assume that the *test substance* can be used *in chemically negligible concentra* for instance because it is labelled If this is not so, the terms $[S_T]/k_{T_A}$ and $[S_T]/k_T$ have to be added to $[S_D]/k_{D_A}$ and $[S_D]/k_{D_B}$ respectively in equ (12) below

Since *uptake* is in fact only measured for the test substance S_T all Q values a tissue/medium ratios for this substance and we can drop the subscript T everywher

The slice/medium ratio Q for the test substance becomes

$$Q = \frac{Q_{max_A}}{1 + \frac{[S_D]}{k_{D_A}} + \frac{[S_I]}{k_{I_A}}} + \quad \frac{\quad}{\quad [S_I]} =$$

$$= \frac{\dfrac{Q_{\max_A}}{1+\dfrac{[S_D]}{k_{D_A}}}}{1+\dfrac{[S_I]}{k_{I_A}\left(1+\dfrac{[S_D]}{k_{D_A}}\right)}} + \frac{\dfrac{Q_{\max_B}}{1+\dfrac{[S_D]}{k_{D_B}}}}{1+\dfrac{[S_I]}{k_{I_B}\left(1+\dfrac{[S_D]}{k_{D_B}}\right)}} \tag{12}$$

In order further to simplify notations we write

$$q_A \text{ for } \frac{Q_{\max_A}}{1+\dfrac{[S_D]}{k_{D_A}}} \quad \text{and} \quad q_B \text{ for } \frac{Q_{\max_B}}{1+\dfrac{[S_D]}{k_{D_B}}}$$

$$k'_{I_A} \text{ for } k_{I_A}\left(1+\frac{[S_D]}{k_{D_A}}\right) \quad \text{and} \quad k'_{I_B} \text{ for } k_{I_B}\left(1+\frac{[S_D]}{k_{D_B}}\right)$$

With this notation (12) becomes

$$Q = \frac{q_A}{1+\dfrac{[S_I]}{k'_{I_A}}} + \frac{q_B}{1+\dfrac{[S_I]}{k'_{I_B}}} \tag{12a}$$

Only Q and $[S_I]$ are known Thus, there are 4 unknowns and in order to estimate them one needs Q values obtained at 4 concentrations of the inhibitor, one of which may be zero Assume that in fact one experiment is run at $[S_I] = 0$, yielding Q_o, then the concentrations used in the other three experiments can be written as C_1, mC_1 and nC_1 and their Q values Q_1, Q_m, Q_n If, finally, one simplifies notations by writing

$$x \text{ for } \frac{C_1}{k'_{I_A}} \quad \text{and} \quad y \text{ for } \frac{C_1}{k'_{I_B}}$$

the system of equations becomes

$$\left.\begin{aligned} Q_o &= q_A + q_B \\ Q_1 &= \frac{q_A}{1+x} + \frac{q_B}{1+y} \\ Q_m &= \frac{q_A}{1+mx} + \frac{q_B}{1+my} \\ Q_n &= \frac{q_A}{1+nx} + \frac{q_B}{1+ny} \end{aligned}\right\} \tag{13}$$

This system gives rise to a quadratic equation

$$y^2 + \frac{II}{I} y + \frac{III}{Q_1 - mQ_m} = 0 \qquad (14)$$

where

$$I = (Q_1 - Q_n)(Q_1 - mQ_m) - (Q_1 - Q_m)(Q_1 - nQ_n)$$

$$II = (Q_1 - mQ_m)\left(Q_1 - Q_0 + \frac{Q_0 - Q_n}{n}\right) - (Q_1 - nQ_n)\left(Q_1 - Q_0 + \frac{Q_0 - Q_m}{m}\right)$$

$$III = \frac{II}{I}(Q_1 - Q_m) + Q_0 - Q_1 - \frac{Q_0 - Q_m}{m}$$

The two roots of the equation are y_1 and y_2 One of them is x and the other y of equation (13) We take y_1 to be the root with $+\sqrt{\ }$ in the solution Solving the system also gives 2 q-values

$$q_1 = \frac{1 + y_1}{y_2 - y_1}[Q_1(1 + y_2) - Q_0] \qquad (15)$$

$$q_2 = Q_0 - q_1$$

q_1 and y_1 belong together

The solution of the system requires 2 cards on a Programma 101 microcomputer The calculations should be performed with 8 decimals

There is an ambiguity in the fact that x and y in equation (13) are roots of the same equation It is therefore not clear which one of the two roots y_1 and y_2 is in fact x of equation (13) and whether q_1 or q_2 is in fact q_A This is of no concern if one only uses the equation for estimation of the affinities of *one* inhibitor But if one studies a series of inhibitors, some of which may have their main affinity to system A and some to B, it is essential to identify those affinities which refer to the same uptake system This can be done with the aid of equation (15), since q_A and q_B do not depend on the inhibitor tested, they should come out with approximately the same value for all inhibitors To take an example we run the first inhibitor and find

$$y_1 = 0\ 001,\ q_1 = 0\ 2,\ y_2 = 0\ 1,\ q_2 = 0\ 8$$

We now arbitrary call the system with y_1 and q_1 the A-system

The next inhibitor may yield

$$y_1 = 0\ 01,\ q_1 = 0\ 75,\ y_2 = 0\ 1,\ q_2 = 0\ 25$$

The q_2-value of 0 25 shows us that for this inhibitor it is y_2 and q_2 that belong to the A system

This identification becomes impossible if q_A and q_B are equal or nearly equal There is a conflict here in the interest of precision they should be reasonably similar (see below), in the interest of identification, they should be reasonably dissimilar

The relative constancy of q_A and q_B in a series of experiments with the same test substance and the same depressor but different inhibitors is a check on the experiments If q_A and q_B come out very far from the usual values, something has gone wrong

The formulae have been derived for the case with a depressor S_D present It would have been the same if a chemically not negligible amount of test substance had been used As is evident from equ (12) one does not obtain absolute values for the affinities $\frac{1}{k_{I_A}}$ and $\frac{1}{k_{I_B}}$ under these conditions, the two affinities are divided by two different but constant factors The situation is analogous to that discussed for the simple uptake system If one needs absolute affinities, one has to run the depressor S_D as inhibitor S_I and use no extra depressor This gives a pair of k_{I_A} and k_{I_B} for the depressor and these can be used as k_{D_A} and k_{D_B} in equ (12) Similarly, if there is an appreciable concentration of test substance S_T, one has to estimate k_{T_A} and k_{T_B} from a separate experiment with the test substance itself in the role of inhibitor

3 *Imperfect input data*

Uptake studies like all other measurements give data with random errors It is therefore not sufficient that a formula can be derived that gives the correct answer from perfect input data, one also wants to know how sensitive the result is to imperfections of the input data and how to design the experiment so as to reduce this sensitivity This will now be discussed

a Choice of a test substance for simultaneous studies

If one wants a similar degree of precision in the affinity values obtained for the two systems, q_A and q_B have to be reasonably similar If they are very different, one system contributes only little to the uptake, its uptake will be swamped by random errors in the uptake of the other and the values obtained for the affinity to the minor system will be uncertain If there is a choice, the test substance S_T should be selected with this in mind If there is no suitable test substance, a suitable depressor may perhaps be found to reduce the contribution of the dominant system to the uptake of test substance towards maybe 70 percent For reasons explained, the two contributions should not be too close

b Simultaneous or successive study of the two systems

It should be remembered that a 100-fold increase in concentration of an inhibitor is needed to bring uptake of the test substance by a single system down from ~91 % of Q_{max} to ~9 % of Q_{max} Hence, if the ratio between the affinities of an inhibitor to the two systems is well under 10^2 there will be appreciable overlap between the concentration ranges of inhibitor within which the main part of the depression

two systems occurs If the ratio between the affinities is considerably higher the conditions may be such that one system can be studied at a time, with the other either virtually suppressed or virtually unaffected Conventional graphical methods can then be used Before studying any one inhibitor quantitatively, it is therefore advisable to find out in a pilot experiment at what concentration uptake of the test substance is depressed by about 10 % and how much is needed for about 90 % suppression If this range is of the order of 10^3 or more, it might be better to study each component separately

c Choice of concentration C_1, mC_1 and nC_1

For simultaneous determinations, it is evident from symmetry that one concentration of inhibitor should correspond to each steep region in the uptake-concentration curve of each system This fixes the values of C_1 and nC_1 The middle concentration mC_1 should be about $C_1\sqrt{n}$

d Averaging results

Equations (14) and (15) were tested in the following way Synthetic input data were calculated using equation (12) and assumed values of k_{I_A}, k_{I_B}, k_{D_A}, k_{D_B}, q_A and q_B Many such combinations were used, with or without a depressor S_D Assumed concentrations C_1, mC_1 and nC_1 were spaced according to the directions just given and ideal values of Q_0, Q_1, Q_m and Q_n calculated A size of error $E = 0\ 05\ Q_0$ was then decided upon This size error corresponds to a *very* successful uptake experiment Then, the ideal Q values, Q_1, Q_m and Q_n (but not Q_0) were made erroneous by adding to them $+E$, $-E$ or 0 in all possible combinations For each of the 27 possible combinations equation (14) was solved and k_{I_A}, k_{I_B}, q_A and q_B tabulated

The results varied widely with the error combination Taking the series of 27 error combinations as a set of replicates, it was obvious that averaging the results did not always produce a value close to the true one under certain conditions a very considerable bias was evident The presence of a depressor was helpful under certain conditions and harmful under others The only recommendation resulting from these extensive tests is, that one should average Q-values from replicate experiments and only then solve equation (14), not use equation (14) on several sets of (poor) input data and then average the results

If there is enough tissue for the experiment to be run at more than 4 concentrations, the opportunity might be taken to obtain an estimate of how well the data of a specific experiment fit the assumption of a 2 component system With 4 equations corresponding to 4 Q-values a fit is always obtained and no test of the assumption is possible If at least one extra concentration and Q value are available, curve fitting by the least-squares method gives an estimate of k_{I_A} k_{I_B}, q_A and q_B and also a sum of squares of deviations which might be useful in deciding which experiments to put reliance on

Several routines for curve fitting are available and are beyond the scope of this paper

Concluding comment

Throughout this paper, the derivation of the formulae is based on the assumption of first order reaction kinetics at the first step of the process which leads to accumulation in the tissue As is well known, formally identical mathematics are valid for reversible adsorption to a surface, for reversible binding to, for instance, a specific protein in the cell and for similar situations There is a difference, however With the present assumption one would expect the mathematics to be applicable especially to short experiments, in which the tissue/medium ratio reflects velocity of uptake With long incubation time, this may not be true For the case of adsorption, especially to an intracellular binding site one would, on the contrary, have to wait for steady state If the experiment is too short, other factors than competition for the binding site may be limiting

The present derivation also bears an obvious similarity to enzyme kinetics In this connection the reader must be warned of a trap when passing from the one field to the other While the tissue/medium ratio achieved in unit time is $Q = V/[S]$, Q_{max} is *not* $V_{max}/[S]$ The reason is of course that Q_{max} and V_{max} are reached at quite different concentrations

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List of symbols

α_1 α_2	intrinsic velocities of uptake
A B	subscripts indicating independent but overlapping uptake systems in the tissue
C_1	lowest concentration of inhibitor
D	subscript indicating the depressor a competitor used in fixed concentration and not analyzed
E	error
F	free receptor fraction $= [R]$ $[r]$
I	subscript indicating inhibitor a substance that competes for uptake is used in several concentrations but is not analyzed
k k_2	dissociation constants of RS_1 RS_2 = inverse affinity
k'_{I_A}	$k_{I_A}(1+[S_D]/k_{D_A})$ Similarly for k'_{I_B}
m n	factors
P	the sum of all $[S]$ k except $[S_I]$ k_I
Q	tissue/med um or slice/medium concentration ratio (actively accumulated
Q_{max}	maximum tissue/medium ratio (reached at vanishingly small concentrat on
q_A	$Q_{max_A}/(1+[S_D]$ $k_{D_A})$ Similarly for q_B
$[R]$	molar concentrations of unbound receptor in tissue
$[r]$	receptor in tissue
$[RS_1]$ $[RS_2]$	in tissue of receptor-substance-complex RS RS
S_1 S_2	substances
$[S_1]$ $[S_2]$	molar concentrations of S_1 S_2 in medium
T	subscript indicating test substance which is taken up and analyzed
v	velocity of uptake moles per l tissue and unit time
V_{max}	maximum velocity of uptake (reached at high chemical concentrat on
x	C_1/k'_{I_A}
y	C_1 k'_{I_B}

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The Disappearance of Acetylsalicylic Acid (ASA) in Aqueous Solution from the Cat Stomach and its Influence on the Transmucosal Ion Transport in the Innervated Non-secreting Stomach

By

BERTIL FRENNING

Received 19 March 1971

Abstract

FRENNING, B *The disappearance of acetylsalicylic acid (ASA) in aqueous solution from the cat stomach and its influence on the transmucosal ion transport in the innervated, non secreting stomach* *Acta* physiol scand 1971 *83* 235—246

During instillation of aqueous solutions of acetylsalicylic acid (ASA) into innervated non secreting whole stomach pouches in the cat parallel decreases in the salicylate and hydrogen ion concentrations took place [illegible] of 20—25 [illegible]
Secretion did not affect the a[illegible]
tion on the ionic permeability [illegible]
after an instillation of ASA [illegible]
inst llation of ASA A study [illegible]

[illegible] but 1 expts hemorrhages occurred during the subsequent HCl instillations It is suggested that the permeability increasing effect of ASA is due to loosening of the mucosa with a consequent increase of the extracellular space

The majority of studies of the effects of acetylsalicylic acid (ASA) on the gastric mucosa have been concerned with morphological effects It is well documented that a single dose of ASA can cause macroscopic mucosal erosions and hemorrhages in both experimental animals and human beings (for example see Douthwaite and Lintott 1938, Anderson 1964, Brodie and Chase 1967)

There is divergence of opinion as to the effect of ASA on the secretion of gastric juice In fasted human beings and dogs Schnedorf Bradley and Ivy (1936) found that after exposure of a stomach to ASA the concentration of hydrogen ions secreted

into distilled water instilled into the stomach over a period of 20 min was greater than before exposure to ASA. Lynch, Shaw and Milton (1964) found in cats with innervated, secreting whole stomach pouches that an intragastric instillation of ASA reduced significantly both the acidity and the secretion rate of gastric juice in comparison with control animals. This effect might be secondary to an increased permeability of the mucosa (*cf* Teorell 1947, Öbrink 1948, Nordgren 1963, Davenport, Warner and Code 1964) and need not necessarily imply an inhibition of the secretory process.

The first to study the effect of ASA on the gastric mucosal permeability for ions was Davenport (1964), who found that 2 consecutive 30 min instillations of 20 mM ASA dissolved in an acid test solution (consisting of 100 mM HCl, 15 mM NaCl and 78 mM mannitol) into vagus-denervated stomach pouches in the dog increased the net fluxes of different ions on subsequent instillation of the acid test solution alone. When the ASA containing solution was neutralized with tris buffer and glycine no effect on the ionic permeability was obtained. (It should be pointed out that Schanker *et al.* (1957) tested the absorption of ASA and found that it diminished with increasing pH.) Later Brodie and Chase (1967) found that acid secretion or administration of exogenous HCl was of great importance for the development of macroscopic mucosal lesions ("gastric irritation") after ASA administration. The same authors also reported a 70 % incidence of gastric hemorrhage during instillation of 100 mM HCl into vagus denervated, but not in vagus-innervated, pylorus ligated rat stomachs, a result suggesting that vagotomy might change the susceptibility of the mucosa to acid (Brodie and Chase 1969).

In view of these findings it was considered of interest to make a study of the transmucosal ion transport during instillations of aqueous solutions of ASA without HCl in innervated secreting or non-secreting whole stomach pouches and also to determine in innervated non-secreting whole stomach pouches whether a 30 min instillation of ASA in aqueous solution influenced the ionic permeability of and the release of pepsin from the gastric mucosa.

Methods

Experimental animal

The expts. were performed on cats weighing between 1.5 and 1.2 kg (mean weight 2.5 kg). The cats were fasted for at least 18 h prior to the expts. but had free access to water. Anesthesia was induced with chloroform and maintained with chloralose-urethan (1:10), given i.v. A midline incision was made and the stomach was isolated by means of ligatures around the cardia and pylorus, care being taken not to disturb the blood and nerve supplies to the stomach. A glass cannula was inserted in the pyloric portion of the stomach and the abdomen was then closed. The stomach was rinsed with physiological saline and its secretory state was checked for two or more h. The mean arterial pressure (±SE, n=15) was 148±5 mm Hg at the start of the expts. and 144±6 mm Hg at the end.

General experimental procedure

6 ml of test solution were instilled in all expts. An instant sample was taken within 1 [illegible] Every 15th min the stomach was em[illegible] [illegible] ... [illegible] measured and samples were taken for analys[illegible] [illegible] chloride, sodium and potassium [illegible] [illegible] procedure, which took 2–3 [illegible]

The expts were started with instillation of 170 mM HCl during two or four 15 min periods, ... was 30 or 60 min, except in some expts ... on The HCl instillation was

acid on the gastric mucosa was ...
after the instillation of ASA ...
and so forth (see Fig 3)

Analyses

... with 5 ml redistilled water and titrated ... The coefficient of variation was ±1 %

Chloride was determined electrometrically with silver silver chloride electrodes in the same samples as were used for acidity determination 5 mM $AgNO_3$ was used for titration The ...

... nded ... led ... for ... 10 ... ro- ... %

at 6 0 mM (n = 10)

Pepsin determination Pepsin was determined by a modified Anson technique (Anson and Mirsky 1932/33, Anson 1938) 0 1 ml samples were incubated with 4 5 ml hemoglobin solution, titrated to pH 2 0, for 10 min at 37° C, and the reaction was then stopped with 10 ml of 0 3 M trichloracetic acid After filtration the absorbance was measured at 280 nm with a Zeiss spectrophotometer, PMQ II The standard curves were derived from crystallised pepsin from Sigma Chemical Company, St Louis, Mo, USA The coefficient of variation was ±3 3 % at 30 P U /ml

Results

Absorption of ASA from non-secreting stomachs and from stomachs with "spontaneous" secretion

In the non secreting stomachs 170 mM HCl was first instilled during 60 min, after which an approximately 16 5 mM (range 15 0—18 1) solution of ASA was instilled (corresponding to about 0 5 g ASA dissolved in 200 ml water) for an equal length of time The ASA solutions were prepared at most 6 h before the expts , the ASA being dissolved in redistilled water at 37° C, cooled to room temperature and filtered before the instillation In the stomachs with spontaneous secretion the instillation of ASA solution was given without any preceding HCl instillation Evacuation of the stomach and sampling were performed as described above The results are given in Fig 1 and 2 and Table I

During the instillation of HCl the normal decrease in the hydrogen and chloride ion concentrations was observed as well as an increase in the potassium and sodium concentrations There was no appreciable change in volume During the first 15 min of the subsequent ASA instillation there was a decrease in the salicylate concentration by 10 4 ± 0 6 mM (mean ± S E) During the next 15 min there was a further decrease of 2 7 ± 0 6 mM and in the last 30 min yet a further decrease of 1 2 ± 0 6 mM A simultaneous and essentially equal reduction in the hydrogen ion concentration was observed (see Fig 1 and Table I) The increase in potassium con-

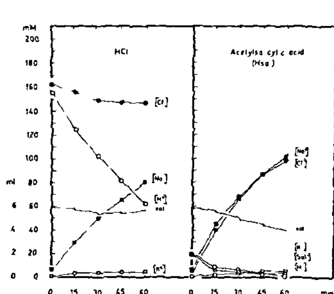


Fig 1

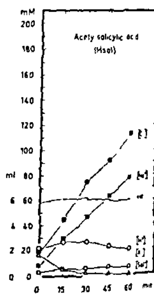


Fig 2

Fig 1 To the left, the result of an instillation of 170 mM HCl into a non-secreting cat stomach To the right, the result of an instillation of approximately 16 5 mM ASA into the same stomach

Fig 2 The result of an instillation of approximately 16 5 mM ASA into a stomach with spontaneous secretion

ABLE I Changes in volume and concentrations of hydrogen, sodium, potassium, chloride and salicylate" ions in aqueous solutions of ASA instilled into cat stomach pouches in 1 h

Exp no	secretory condi-tion	ΔH+ mM	ΔSali-cylate mM	ΔH+-ΔSal mM	ΔNa+ mM	ΔK+ mM	ΔCl- mM	ΔCl (ΔNa+ + ΔK) mM	ΔV ml
1	arrest	—19	—13 3	— 6	+70	+ 4	+ 80	+ 6	—1 7
2	"	—11	—14 5	+ 4	+79	+ 3	+ 83	+ 1	—1 1
3	"	—19	—15 9	— 3	+97	+ 5	+ 94	— 8	—1 2
4	"	—11	—12 5	+ 2	+80	+ 4	+ 83	— 1	—1 6
5	"	—16	—15 5	— 1	+92	+ 6	+104	+ 6	—1 0
Mean ± S E		—15±2	—14 3± 0 6	—1±2	+81±5	+4±1	+89±4	+ 1±3	—1 3± 0 1
6	secretion	+26	—14 9	+41	+66	+ 5	+113	+42	—1 0
7	"	— 1	—15 4	+14	+61	+ 4	+ 78	+13	+0 1
8	"	+73	—14 9	+88	+20	+11	+118	+87	+4 7
9	"	0	—16 3	+16	+71	+ 4	+ 95	+20	—0 7
10	"	+ 7	—14 5	+22	+62	+ 6	+109	+41	+0 2
Mean ± S E		+21±14	—15 2± 0 3	+36±14	+56±7	+ 6±1	+103±7	+41±13	+0 9± 0 9

centration was approximately the same during instillation of ASA as during HCl instillation The sodium concentration increased more rapidly during instillation of ASA than of HCl The sum of the increase in the potassium and sodium concentrations was on the average equal to the increase in chloride concentration There was a mean decrease in volume of 1 3 ml The net result was thus an outward (from the gastric lumen) diffusion of undissociated ASA an inward (into the gastric lumen) diffusion of sodium potassium and chloride ions and water absorption No hemorrhages were observed during these experiments and no macroscopic mucosal erosions occurred

During instillation of ASA in spontaneously secreting stomachs there was a decrease in the salicylate concentration during the first 15 min by 11 2±0 6 mM (mean±S E) During the next 15 min the decrease was 2 7±0 3 mM and during the last 30 min 1 4±0 3 mM

Secretion did not thus appear to influence the absorption of ASA As can be seen in Table I secretion could be present even though the volume decreased during the instillation The mean increase in sodium ion concentration was lower, and the mean increase in chloride ion concentration higher during instillation of ASA into secreting than into non secreting stomachs No hemorrhages or macroscopic mucosal lesions were observed in these expts either

Table I gives the changes in hydrogen ion concentration (ΔH) related to the initial acidity in the instilled ASA solution Since it is probable that one hydrogen ion disappeared at the same time as every salicylate ion *i e* ASA was absorbed (ΔH$-\Delta$Sal) would give a more correct measure of the change in hydrogen ion concentration during the secretion expts This was on the average +36±14 mM and was accompanied by an increase in chloride ion concentration of 41±13 mM over the sum of the increase in potassium and sodium ion concentrations The total increase in concentration of positive ions (ΔH$-\Delta$Sal$+\Delta$Na$+\Delta$K) was 98 mM and this increase corresponded to an increase in chloride ion concentration by 103 mM

Effect of ASA in aqueous solution on the ionic permeability of the gastric mucosa and the duration of this effect

These expts were performed on non secreting stomachs 170 mM HCl was first instilled during 30 min in order to determine the normal ionic permeability The stomach was then emptied and ASA solution was instilled over a similar length of time after which repeated instillations of 170 mM hydrochloric acid were given The results are presented in Fig 3 and 4 and Table II and III

Table II gives the mean changes in concentration of H Cl Na and K ions and volume in 170 mM HCl solutions instilled *before* (HCl) and *after* HCl— HCl$_5$) an instillation of ASA After an installation of ASA significantly larger decreases in hydrogen and chloride ion concentrations were observed ($p < 0.01$ and $p < 0.02$ respectively) and a larger increase in sodium concentration ($p < 0.02$ but no change in the increase in potassium concentration ($p > 0.5$) during instilla

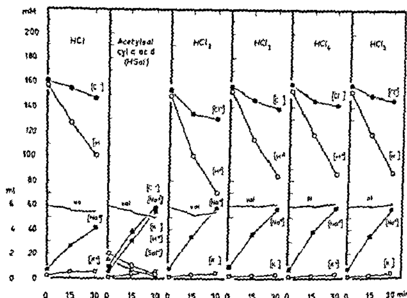


Fig 3 The result of an expt with one HCl instillation before and four consecutive instillations after a 30 min instillation of ASA in aqueous solution

tion of 170 mM hydrochloric acid It should be noted that there was no appreciable difference in change in volume on comparison between HCl_2 and HCl_1 During subsequent HCl instillations the concentration changes were reduced, and during HCl (90—120 min after completion of the ASA instillation), no change in concentration differed significantly ($p > 0.05$) from the initial value During HCl the mean change in volume was -0.2 ml During HCl_3—HCl_5 the corresponding change in volume was $+0.4$ ml It should be noted, however, that only in 2 out of 7 expts did the increase in volume during HCl_3 and HCl_4 exceed 0 5 ml For HCl_5 the corresponding figure was 1 expt out of 4

Since the concept of "exchange diffusion between hydrogen and sodium ions" introduced by Teorell (1933 1939 1947) has been interpreted by a number of authors as meaning that the decrease in hydrogen ion concentration and the increase in sodium concentration necessarily are equal it should be pointed out that consideration must also be taken of the common anion i e of the change in chloride ion concentration (see Teorell 1947 eqns 10 11 and 12) As can be seen in Table II the mean increase in sodium ion concentration was smaller than the corresponding decrease in hydrogen ion concentration during instillation of 170 mM HCl If on the other hand the net decrease in hydrogen ion concentration ($\Delta H - \Delta Cl$) is compared with the summed increase in the sodium and potassium ion concentrations it is seen that these are equal This holds for HCl instillations performed both before and after an instillation of ASA

The effect of ASA on the gastric mucosal permeability for ions was maximal immediately after the ASA instillation Since there was a mean decrease in volume

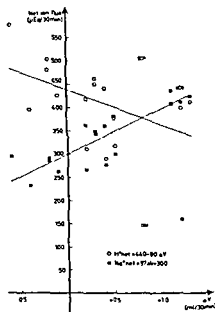


Fig 4 Relationship between the net fluxes of hydrogen ions and sodium ions, respectively, and

TABLE II Changes in … ions during … tween the fir… 16 5 mM AS…

HCl instilla-tions no	n	ΔH+ (mM)	ΔCl- (mM)	(ΔH+— ΔCl-)	ΔNa+ (mM)	ΔK+ (mM)	(ΔNa+ + ΔK+)	ΔV ml
1	7	—50±4	—14±1	—36	+33±2	+2±1	+35	±0 0±0 1
2	7	—81±3	—29±3	—52	+50±2	+3±1	+53	—0 2±0 2
3	7	—65±5	—21±1	—44	+43±5	+3±1	+46	+0 4±0 2
4	7	—64±4	—19±2	—45	+42±4	+2±1	+44	+0 4±0 2
5	4	—64±3	—19±3	—45	+47±4	+2±1	+49	+0 4±0 3

TABLE III The mean net fluxes of hydrogen, chloride, sodium and potassium ions ± SE in the same expts as reported in Table II

HCl instilla tion no	n	H+net (μEq/30 min)	Cl-net (μEq/30 min)	Na+net (μEq/30 min)	K net (μEq 30 min
1	7	—371±23	—118±36	+243±16	+20±3
2	7	—566±26	—231±40	+312±18	+18±2
3	7	—439±33	—102±38	+307±34	+18±1
4	7	—412±25	—100±34	+298±33	+18±2
5	4	—412±37	— 87±38	+332±38	+18±1

stillation of 20 mM SA in 1 mM HCl the inflow of plasma and interstitial fluid did not differ significantly from the control value The most likely explanation to volume increases seems to be that acid secretion had been provoked Acid has been reported after administration of ASA to unstimulated stomachs (Schnedorf, Bradley and Ivy 1936) It is possible that acid secretion may be initiated by polypeptides liberated by peptic digestion An ASA induced histamine release from the mucosa might also stimulate acid secretion (*cf* Davenport 1966, Johnson and Overholt 1967) In an attempt to determine the effect of volume changes during the periods HCl_3—HCl_5 the net fluxes of sodium and hydrogen ions, respectively were plotted against the corresponding changes in volume, see Fig 4 The regression lines indicate that the efflux of hydrogen ions was inversely, and the influx of sodium ions directly proportional to the volume increase obtained Both these changes could have been caused by acid secretion in combination with an increased mucosal permeability As regards sodium ions an inflow of plasma and interstitial fluid could have contributed to the results, but in the light of Davenport s findings (1966) such a contribution would probably be unimportant Thus it is most probable that the reduction of the net effluxes of hydrogen and chloride ions observed during HCl_3—HCl_5 was only apparent and caused by secretion of HCl The high net influxes of sodium ions found in the instillation periods HCl_3—HCl_5 were thus probably the results of an increased hydrogen sodium "exchange' diffusion masked by HCl secretion

ASA instillation gave rise to a marked increase in the release of pepsin from the *mucosa during subsequent instillations of hydrochloric acid This might be of im*portance for the development of gross mucosal lesions and bleeding from the mucosa As irrigation of untreated gastric mucosa with acid solutions of pepsin did not influence the mucosal permeability (Davenport 1965) it is probably not primarily related to the permeability increasing effect of ASA

It has been shown on dogs that the gastric mucosal blood flow, measured by aminopyrine clearance, increased during instillation of ASA (Augur 1970) His results do not however, indicate a relationship between the mucosal blood flow and the increased ionic permeability subsequent to the ASA instillation Thus the mechanism underlying the permeability increasing effect of ASA remains unknown though it is probable (as was suggested by Martin in 1963) that it is related to an intracellular accumulation of the drug Such an accumulation would cause intracellular hypertonicity and swelling of the mucosal cells Such a swelling might increase the extracellular space It has been postulated earlier that the permeability increasing effect of acetic acid could be explained in this [illegible] Flemstrom and Frenning 1968 Flemstrom (1971) presented data which stron[illegible] [illegible] a temporary intracellular accumulation of acetate, p[illegible] and L(+ [illegible] is probable, though not proven, that also salicylat[illegible] [illegible]ily ac[illegible] mucosal cells Morris *et al* (1967) demonstr[illegible] the [illegible] the corpus per[illegible]tion of the stomach [illegible] than in other tissues (muscle, blood, kidr[illegible]

Instillation of ASA gives rise to a significant increase in the DNA content of gastric irrigation fluid indicating loss of cells from the mucosa (Croft 1966, Croft and Wood 1967) This cell loss can possibly be explained as the result of a very large intracellular accumulation of the drug Whatever the underlying mechanism it would however, seem reasonable to assume that a process which ends with cell loss from the mucosa will also give rise to a more extensive loosening of the mucosa, with a consequent increase of the extracellular space It has been shown by electron microscopic method that calcium depletion in the bullfrog gastric mucosa, earlier found to increase the mucosal permeability (Forte and Nauss 1963), gave rise to disorganization or separation within the intercellular junctions (Sedar and Forte 1964) Öbrink and Waller (1965) have given experimental support to the view that hydrogen ions disappears from the stomach via intercellular diffusion Thus there are experimental results which points to the probability that the ion 'exchange' across the mucosa takes place extracellularily (As regards potassium the situation is more complicated, *cf* Nordgren 1963) An increase of the extracellular space might thus explain the permeability increasing effect of ASA

Effects of ASA on the intermediary metabolism have been known since 1943, when von Euler and Ahlstrom showed that salicylate inhibited glucose and lactate dehydrogenases *in vitro* Inhibition of several dehydrogenases in the Krebs cycle has since been demonstrated (Kaplan Kennedy and Davis 1954, Bryant, Smith and Hines 1963, Hines and Smith 1964) Inhibition of transaminases and an uncoupling effect on oxidative phosphorylation reactions have also been found (Huggins, Smith and Moses 1961, Brody 1956) An accumulation of salicylate would thus appear to cause an inhibition of the energy producing processes in the cells This might apart from other effects also contribute to a cellular swelling due to failure of the ion pumps which all cells are supposed to possess (*cf* Robinson 1965)

I am greatly indebted o Mrs Lena Westlund for her invaluable technical assistance Financial support was given by the Medical Faculty of the University of Uppsala, the Swedish Society for Medical Research and the Swedish Medical Research Council

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Studies of Monosaccharide Permeability of Arterial Tissue and Intestinal Smooth Muscle; Effects of Insulin

By

HANS J ARNQVIST

Received 24 March 1971

Abstract

ARNQVIST, H J *Studies of monosaccharide permeability of arterial tissue and intestinal smooth muscle, effects of insulin* Acta physiol scand 1971 *83* 247—256

[illegible]

tion of unlabelled D glucose determined by an enzymatic method was constant during the same incubation times and corresponded to a distribution in the extracellular space It seems probable therefore that the membrane permeability of D glucose is rate limiting for the metabolism of glucose in the smooth muscle cells

Insulin (0 1 U/ml) increased moderately the accumulation of C^{14} labelled D glucose in rat aorta and rabbit colon and the accumulation of D xylose C^{14} in rabbit colon

Patients with diabetes mellitus are more prone to obstructive arterial diseases than other persons As the development of degenerative arterial diseases probably is associated with changes of the metabolic activity of the vascular wall (Kirk 1968 Whereat 1967 Adams and Bayliss 1969) it is of great interest to know whether insulin influences the metabolic activity of arterial tissue Few studies on this subject have been reported in the literature and the results have been partly controversial

Wertheimer and Ben Tor (1962 found that the uptake of D glucose and D xylose in rat aorta was stimulated by insulin Urrutia Beavan and Cahill (1962) carefully freed rat aorta from adventitia and perivascular adipose tissue They found no effect of insulin on the glucose oxidation or incorporation of glucose carbon into glycogen and lipid but the glucose oxidation in the perivascular adipose tissue was strongly stimulated by insulin Lundholm and Mohme Lundholm (1963) investi-

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KH_2PO_4, 25 $NaHCO_3$
1 mg glucose/ml was added to the preincubation medium The buffer was gassed with a mixture of 95 % O_2 and 5 % CO_2 for approximately 1 h and the incubation flasks were then gassed for 20 s immediately before the incubation The flasks were sealed with tightly fitting rubber stoppers

Chemicals D glucose $C^{14}(U)$, D xylose-$C^{14}(U)$, sorbitol $C^{14}(U)$ and sucrose $C^{14}(U)$ were obtained from the Radiochemical Centre, Amersham, England Instagel and Soluene were commercial preparations of Packard Instrument Company Inc Hexokinase and glucose 6 ... r, Mannheim A 10-fold recrystallized

... *umulation* Each flask contained 0 2 ... 11 1 mM D glucose When the tissue had been ... was ... t ll ... am ... e external standard teqnique The counting efficiency ...

stagel and the activity of the isotope ...
Duplicate samples were always counted ...
as distribution in per cent of the wet tissue weight 100 × cpm mg wet tissue weight/cpm per μl incubation medium

Chromatography To test if D xylose C^{14} was metabolized tissue extracts were prepared and checked for xylose metabolites by thin layer chromatography After an incubation period of 180 min in 13.3 mM D xylose C^{14} the tissue was homogenized in 6 % perchloric acid and neutralized with K_2CO_3 The protein free extract was spotted on precoated cellulose (Avicel) thin layer plates 20 by 20 cm and 250 μ thick (Analtech Inc)
The plates were developed ...
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To ...
weight at 100 C The total tissue water was then calculated as per cent of the wet tissue weight

Extracellular space Sorbitol C^{14} or sucrose C^{14} was added to the incubation medium in a final concentration of 0 5 mM The distribution of these isotopes in the tissue was calculated as for D xylose C^{14}

Enzymatic determination of D glucose distribution After the incubation the tissue was washed in buffer for 10 sec and then frozen rapidly at −80° C in frigen 12 containing solid CO_2 The frozen tissue was homogenized in 6 % perchloric acid and after neutralisation with K_2CO_3 the glucose content of the tissue extract was analysed by the hexokinase glucose 6 phosphate dehydrogenase method (Slein 1962) Duplicate samples from the incubation medium were taken and assayed for glucose by the same method The tissue distribution was calculated as per cent, 100 × glucose concentration per mg tissue wet weight/glucose concentration per μl incubation medium

Results

A *Rat and rabbit aorta*

Accumulation of D glucose C^{14} in rat aorta, effect of insulin As can be seen in Table I the accumulation of D glucose C^{14} in rat aorta was stimulated by insulin The effect was small but statistically significant after incubation periods of 120 and 180 min

TABLE I Effect of insulin on D glucose C^{14} accumulation in rat aorta Insulin was added in a concentration of 0 1 U/ml medium and the D glucose concentration in the medium was 11 1 mM The glucose accumulation is calculated as mg 10^{-2}/100 mg tissue wet weight n = number of rats p = probability that the effect was due to chance The values are given ±SE

Incubation time (min)	Control	Insulin	Increase with added insulin
120 n = 12	8 0±0 4	8 9±0 3	0 9±0 3 p < 0 01
180 n = 5	9 1±0 3	10 1±0 1	1 0±0 2 p < 0 01

Time course of D xylose-C^{14} accumulation in rat aorta and rabbit aorta The effect of insulin on the glucose-C^{14} accumulation in rat aorta may indicate an effect on the membrane transport of monosaccharides The accumulation of D xylose-C^{14} was therefore studied in rat and rabbit aorta at incubation periods varying between 5 and 180 min (Fig 1) To estimate the extracellular space the distribution of sorbitol-C^{14} was also investigated The accumulation of both D xylose C^{14} and sorbitol C^{14} was very rapid during the first 5—30 min After this first phase the D-xylose-C^{14} accumulation continued to increase but at a slower rate, while that of sorbitol-C^{14} was practically constant After the first 15—30 min the D xylose C^{14} space clearly exceeded the sorbitol-C^{14} space This indicates that D xylose is distributed in the intracellular space and that the intracellular distribution takes place at slower rate than the extracellular distribution

Effect of insulin on D-xylose-C^{14} accumulation in rat aorta The distribution of D xylose C^{14} increased with time There was a tendency to a higher D xylose C^{14} distribution in the rat aortas treated with insulin (Fig 2), but the difference was not significant (after an incubation period of 180 min $0\,05 < p < 0\,10$)

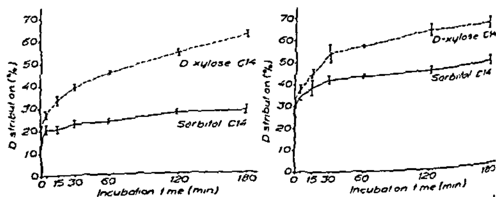
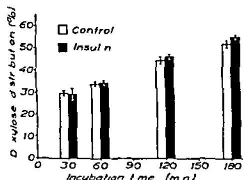


Fig 1 Distribution of D xylose-C^{14} 13 3 mM and sorbitol C^{14} 0.5 mM in rat (left) and rabbit aorta (right), expressed as per cent of the wet tissue weight Sorbitol space was determined in the presence of 13 3 mM unlabelled D xylose Mean ± SE (n = 5)



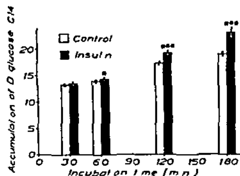


Fig 2 Fig 3

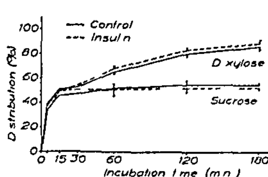
Fig 2 Effect of insulin (0 1 U/ml) on the accumulation of D xylose C^{14} in rat aorta. The concentration of D xylose in the incubation medium was 13.3 mM The accumulation is expressed as distribution in per cent of wet tissue weight. Mean ± SE (n 3—10)

Fig 3 Effect of insulin (0 1 U/ml) c
from rabbit colon The glucose conce
lated as 10^{-2} mg/100 mg tissue wet we
* = $p < 0\ 05$, ** = $p < 0\ 01$ *** =

Total tissue water in rabbit and rat aorta Total tissue water was investigated after preincubation and after incubation with either glucose 11 1 mM or D xylose 13 3 mM For rabbit aorta incubated in D xylose the total tissue water was 71 1 ± 0.5 % of the wet tissue weight after 10—30 min of preincubation and 75 3 ± 0 8 % after 180 min of incubation The increase 4 2 ± 1 2 % (n 5) was statistically significant ($p < 0\ 05$) Rat aorta also showed an increase in total tissue water when the incubation medium contained D xylose After preincubation for 10 min the total tissue water was 67 3 ± 0 7 % (n = 5) and there was an increase of 4 2 ± 0 6 % ($p < 0\ 01$) after an incubation period of 180 min With glucose present in the incubation medium there was an increase in total tissue water of 1 5 ± 4 % (n 10 $p < 0\ 01$) for rabbit aorta and 1 3 ± 0 6 % (n = 5 $p < 0\ 10$) for rat aorta The increase in total tissue water during the incubation period of 180 min was thus greater when D xylose was present in the incubation medium than when D glucose was used When rabbit aorta was incubated for 180 min without substrate the total tissue water increased by 4 2 ± 7 4 % (n = 10 $p < 0\ 02$) D xylose is not metabolized by vascular tissue (see methods) The increase in total tissue water after D xylose may therefore be due to substrate depletion

B *Rabbit colon*

Effect of insulin on D glucose C accumulation in rabbit colon This effect was studied at incubation periods varying between 30 and 180 min (Fig 3 Addition of insulin resulted in stimulation of the D glucose C^{14} accumulation after 60 min With increasing incubation periods the difference in D glucose-C^{14} accumulation between insulin treated tissues and controls grew more pronounced Compared with rat aort



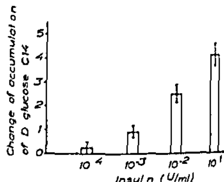


Fig 4

Fig 5

Fig 4 Comparison between the accumulation of D xylose C^{14} (13 3 mM) and sucrose C^{14} (0 5 mM) in smooth muscle from rabbit colon with and without added insulin (0 1 U/ml) In the expts with sucrose C^{14} unlabelled D xylose was added in a concentration of 13 3 mM The accumulation of D xylose C^{14} and sucrose C^{14} is calculated as distribution in per cent of the wet tissue weight *At incubation periods of 120 and 180 min the increase in D xylose* C^{14} accumulation was significant ($p < 0\ 01$)

Fig 5 Increase of D glucose C^{14} accumulation in smooth muscle from rabbit colon at insulin concentrations varying between 10^{-4} and 10^{-1} U/ml medium D glucose C^{14} accumulation is calculated as 10^{-4} mg/100 mg wet weight Mean ± SE (n = 10)

the D glucose C^{14} uptake was much higher in rabbit colon after an incubation period of 180 min (rat aorta 9 1 ± 0 3 and rabbit colon 19 1 ± 0 4 × 10^{-} mg/100 mg wet tissue weight) The effect of insulin was also more pronounced in rabbit colon

Accumulation of D xylose-C^{14} in rabbit colon effect of insulin From Fig 4 it is evident that on incubation with 13 3 mM D xylose C^{14} there was a rapid accumulation of D xylose C^{14} during the first 20—30 min, followed by a slower rise which continued for at least 180 min In the presence of 0 1 U/ml of insulin the D xylose C^{14} accumulation was significantly greater after 120 and 180 min The insulin effect was moderate however For comparison the accumulation of C^{14} labelled sucrose (0 5 mM sucrose C^{14} in the presence of 13 3 mM unlabelled D xylose) is also shown in Fig 4

Dose response effect of insulin The dose response relationship was studied in rabbit colon at insulin concentrations of 10^{-1} – 10^{-4} U/ml of medium (Fig 5) Arvill and Ahren (1967) found that insulin could become inactivated during the incubation period The activity of insulin in the incubation medium was therefore investigated When the incubation of rabbit colon was terminated a piece of rat diaphragm was incubated in the same medium and the D glucose C^{14} accumulation measured At the lowest concentration 10^{-4} U/ml insulin had no measureable effect on the D glucose C^{14} accumulation in rabbit colon although insulin activity in the medium was demonstrable by the rat diaphragm assay In the dose range 10^{-3} 10^{-1} U/ml the effect of insulin rapidly increased with increasing concentrations

Extracellular space in rabbit colon The extracellular space was estimated by measuring the distribution of 0 5 mM sucrose C^{14} or 0 5 mM sorbitol C^{14} (Table

TABLE II Sucrose C^{14} and sorbitol C^{14} spaces in smooth muscle from rabbit colon at varying incubation times Sucrose C^{14} and sorbitol C^{14} were used in a concentration of 0.5 mM in the presence of unlabelled D glucose 11.1 mM or D xylose 13.3 mM The spaces are calculated as per cent of the wet tissue weight Mean ± SE

Incubation medium	Insulin 0.1 U/ml	Incubation times (min) 5	15	30	60	120	180
Sorbitol C^{14} D-glucose (n = 5)	0	30.6±2.9	40.3±4.9	43.4±3.9	41.6±4.7	43.1±2.3	42.2±1.7
	+	29.5±1.7	43.1±4.3	44.3±4.7	40.3±2.5	41.3±2.3	43.8±2.5
Sucrose C^{14} D-glucose (n = 5)	0	30.8±2.9	43.5±3.2	49.7±2.5	44.7±3.1	45.8±4.0	47.9±4.0
	+	37.5±2.6	38.7±2.2	44.2±2.3	41.0±1.7	46.1±2.4	48.6±2.1
Sucrose C^{14} D-xylose (n = 5)	0	34.1±2.0	46.1±2.8	47.9±2.5	52.2±3.8	55.3±3.7	55.1±1.1
	+	33.1±2.4	49.2±3.0	51.1±1.6	51.3±5.6	52.8±3.5	52.6±1.7

II) The sucrose C^{14} space and sorbitol C^{14} space were studied during incubation periods varying between 5 and 180 min in the presence of unlabelled D glucose at a concentration of 11.1 mM The sucrose C^{14} space was also determined during the same incubation periods in the presence of unlabelled D xylose at a concentration of 13.3 mM The expts were performed with and without added insulin There was always a rapid increase in the distribution of sucrose C^{14} and sorbitol C^{14} during the first 30 min of incubation. During the rest of the incubation period the spaces remained quite constant No effect of insulin was seen No differences was found when the distributions of sucrose C^{14} and sorbitol C^{14} were determined on tissues from the same animals in the presence of D glucose (11.1 mM) at incubation periods of 30 60 and 180 min

Time course of D glucose C^{14} accumulation in rabbit colon The D glucose C^{14} accumulation was studied at incubation periods varying between 5 and 180 min In Fig 6 the D glucose C^{14} accumulation is calculated as distribution in per cent of the wet tissue weight i e the space which the accumulated D glucose C^{14} would have occupied if it were not metabolized The D glucose C^{14} space" is compared with the distribution of sorbitol C^{14} During the first 30 min the accumulation of both sorbitol C^{14} and D glucose C^{14} was very rapid After this period this sorbitol C^{14} space remained constant The D glucose-C^{14} space increased steadily but at a much slower rate than during the first 30 min.

Distribution of unlabelled D glucose in rabbit colon In order to find out if there was any free intracellular glucose the tissue concentration of D glucose was determined by the hexokinase glucose 6-phosphate dehydrogenase method and the glucose distribution was calculated The distribution of glucose in a concentration of 11.1 mM was determined at incubation times of 60 120 and 180 min (Fig 7) During this time it was found to be constant Insulin had no effect on the glucose distribution In Fig 7 the distribution of free glucose is compared with the sorbitol space and the D glucose C^{14} accumulation (calculated as distribution) The figure

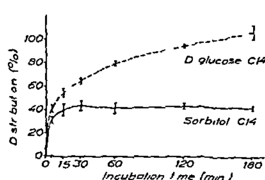


Fig 6

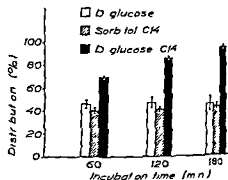


Fig 7

Fig 6 The time courses of the accumulation of D glucose C^{14} and sorbitol C^{14} in rabbit colon In both expts the glucose concentration was 11 1 mM Sorbitol C^{14} was added in a concentration of 0 5 mM The accumulation was calculated as distribution in per cent of the wet tissue weight (Mean ± SE) Each point represents the mean of 4—5 observations

Fig 7 Comparison between the accumulation of free D glucose D sorbitol C^{14} and D glucose-C^{14} in smooth muscle from rabbit colon The accumulation of D glucose C^{14} and sorbitol C^{14} were calculated as distribution in per cent of the wet tissue weight (100 × cpm per mg tissue wet weight/cpm per μl incubation medium) The distribution of free glucose was calculated as 100 × glucose concentration per mg tissue wet weight/glucose concentration per μl incubation medium

shows that the sorbitol space and the glucose space remained constant and were almost equal The small difference is explained by the fact that the exp was performed on muscle from different groups of animals The distribution of D glucose C^{14} increased steadily The distribution of D glucose C^{14} is a measure of both the glucose and the metabolized glucose carbon These findings would seem to indicate that metabolized glucose accumulates intracellularly while free glucose is restricted to the extracellular space

Total tissue water in rabbit colon The total tissue water was determined after preincubation and after incubation for 180 min with glucose in a concentration of 11 1 mM with or without insulin In the same way the total tissue water was determined with D xylose in a concentration of 13 3 mM After preincubation for 10—30 min the value was 81 3 ± 0 5 % of the wet tissue weight The same value was found after 180 min incubation with glucose In the expts with D xylose the total tissue water was 82 1 ± 0 3 % after the preincubation period After 180 min incubation in D xylose it was slightly higher 82 9 ± 0 4 The difference, 0 8 ± 0 3 was statistically significant ($p < 0.05$) No effect of insulin on the total tissue water was seen

Discussion

In this investigation insulin was found to have a stimulating effect on the accumulation of D glucose C^{14} in rat aorta The rat aorta was carefully freed from adventitia and perivascular tissue The effect of insulin cannot therefore be explained by stimulation of extraneous insulin sensitive tissue Urrutia *et al* (1962) pointed out the importance of using a rat aorta preparation free from extraneous tissue They studied the effect of insulin on the metabolism of C^{14} labelled D glucose and found

no effect of insulin on the oxidation of D glucose C^{14} to CO or incorporation of D-glucose C^{14} into glycogen and lipids The method used in this study measured the total accumulation in tissue of D glucose C^{14} and its metabolites As the effects of insulin on the glucose metabolism in rat aorta are comparatively small, difference in the sensitivity of the methods may explain the discrepant results The D xylose C^{14} distribution in rat aorta steadily increased with time but was not significantly changed by insulin In skeletal muscle insulin increases the distribution of D xylose by stimulating the membrane transport (Kipnis and Cori 1957)

Insulin stimulated the accumulation of D glucose C^{14} and D xylose C^{14} in a smooth muscle preparation from rabbit colon The dose response relationship was investigated on glucose accumulation to determine whether insulin had any effect in low concentrations In this study a small effect was noted at a concentration of 1000 μU/ml but no effect was observed at 100 μU/ml By immunoassay the blood concentration of insulin in rabbits has been found to vary between 10—50 μU/ml (Nijjar and Perry 1970)

In smooth muscle the size of the extracellular space varies with the molecules used as extracellular markers (Barr and Malvin 1965), and for estimating this value in smooth muscle sorbitol is considered to be a suitable substance (Casteels 1970) In this study both the sorbitol C^{14} and the sucrose C^{14} space were analyzed in a smooth muscle preparation from rabbit colon The spaces were of equal size when studied on the same animals After an initial increase during an incubation time of 30 min the spaces were constant for a period of at least 150 min

It is evident from Fig 4 and 6 that the time course of D glucose C^{14} and D xylose C^{14} accumulation in rabbit colon was quite different from that of sorbitol C^{14} and sucrose C^{14} During the initial 15—30 min the distributions were very similar and it seems probable that during this time the substances were distributed in the extracellular space After 30 min there was a continuous increase of the tissue activity both after treatment with D glucose C^{14} and D xylose C^{14}, whereas with sorbitol C^{14} and sucrose C^{14} it was constant With D xylose C^{14} and sorbitol C^{14} the same result was obtained in rabbit and rat aorta These findings indicate a specific transport system in the smooth muscle cells for glucose and xylose which cannot be used by sucrose or sorbitol The distribution of free glucose in smooth muscle determined by an enzymatic method was of the same magnitude as that of sorbitol C^{14} It seems probable therefore that free glucose was confined to the extracellular space and that there was no accumulation intracellularly This would seem to mean that the transport system is rate limiting for the glucose metabolism of rabbit colon smooth muscle These findings are in contrast to those of Yalcin and Winegrad (1962), who compared the spaces of raffinose and glucose in rabbit aorta after an incubation period of 30 min They found that the glucose space exceeded the raffinose space and concluded that there was free glucose in the intracellular space and that membrane transport was not rate limiting for glucose metabolism in this preparation The time course of D xylose accumulation in rat and rabbit aorta (Fig 1) indicates that in these tissues the cell membrane has a regulating action on the rate

of tissue distribution of monosaccharides It seems probable, therefore, that the mem brane transport of glucose may influence the glucose metabolism in vascular tissue too Further studies are, however, necessary to clarify if the membrane transport of glucose is rate limiting for its metabolism in vascular tissue

Insulin increased both the accumulation of C^{14}-labelled glucose and D xylose in smooth muscle from rabbit colon As D xylose is not metabolized by the rabbit colon smooth muscle the insulin effect on the D xylose-C^{14} distribution indicate an activation of the membrane transport of D xylose The insulin effect in rabbit colon was rather weak, however, in comparison to its effect in skeletal muscle

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Uptake of Serotonin in Blood Platelets in vitro. II: Further Studies on the Effects of Small Anions

By

O Lingjærde, Jr

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Abstract

Lingjærde, O Jr *Uptake of serotonin in blood platelets in vitro II Further studies on the effects of small anions* Acta physiol scand 1971 *83* 257—268

According to previous reports, serotonin (5HT) uptake in human blood platelets *in vitro* requires the presence of chloride Chloride can be replaced by other small univalent, but unphysiological anions, such as (in decreasing order of effectiveness) bromide, nitrite, iodide, nitrate and formate, However, the effect of nitrite, especially, is kinetically different from tha[illegible] pe kinetics, inc[illegible] the effects of [illegible] rite can be exp[illegible] ie so-called "a[illegible] site" of the carrier, also has an effect on a hypothetical "activating site" This simple model cannot, however easily explain all the complex interactions between chloride and nitrite It is also reported that preincubation with different anions affects the uptake rate to very varying degrees, and that the anions have somewhat different effects on spontaneous 5HT outflux

Blood platelets, as well as some neurons, can take up and store relatively large amounts of serotonin (5 hydroxytryptamine, 5HT) Two different mechanisms are involved in the transport through the platelet plasma membrane one "active" mechanism, which obeys a Michaelis-Menten type kinetics, and one "passive" mechanism, which has the characteristics of passive diffusion Once inside the plasma membrane, 5HT is rapidly taken up into special storage granules (Pletscher 1968)

The 5HT uptake mechanism in platelets (which, in this respect, may also be considered as a model of serotoninergic neurons) has been studied by a number of workers (see Pletscher 1968), but many details of the mechanism are still not known Previous studies from this laboratory have shown that the "active" uptake of 5HT in human blood platelets requires the presence of extracellular chloride (Lingjærde 1969, 1971), and it has been suggested that chloride has to be bound to the 5HT carrier in order that the translocation of the carrier substrate complex, from the outside to the inside of the membrane, can take place (Lingjærde 1971)

In vitro, chloride can be replaced by other small univalent anions such as bromide nitrite, and nitrate (Lingjærde 1969) In the present communication the effects of anions other than chloride will be dealt with in more detail, and it will be shown that these effects may throw some new light on the 5HT uptake mechanism

Materials and methods

The materials and methods were described in detail in a previous communication (Lingjærde 1971) Platelets from healthy persons were isolated by differential centrifugation and resuspended in portions of 2 ml 55 mM phosphate buffer, pH 7 3 (if not otherwise stated The sodium salts of the different anions under study were added in concentrations as specified in the description of each experiment Sodium sulphate was used when appropriate to replace the sodium salt of the anion under study, since the sulphate anion was found to be inert with regard to 5HT uptake The potassium concentration was kept constant at 5 mM and the sodium concentration at approximately 175 mM The total osmolarity was kept near to isotonicity *i e*, at about 300 mM Control experiments showed that this simple artificial incubation medium (with chloride as the active anion) gave about the same initial uptake rate per platelet as was found in platelet rich plasma (Lingjærde 1970) In accordance with previous reports glucose was found not to influence the uptake rate (Hughes and Brodie 1959 Stacey 1961), and was therefore deleted

If not otherwise stated the mixtures were preincubated for 5 min at 37° C for temperature equilibration and thereafter incubated with ^{14}C 5HT for 5 min The ^{14}C 5HT was always added in a volume of 20 μl *i e* 1 % of the incubation volume The incubation was stopped by transferring the test tubes to ice water Thereafter the platelets were isolated by centrifugation and the 5HT released by deep freezing with distilled water The platelet bound ^{14}C 5HT was measured by liquid scintillation counting Blank values were obtained by adding ^{14}C 5HT to control samples kept in ice water and internal standards were prepared from samples to which ^{14}C 5HT was added together with the distilled water prior to freezing

The outflux experiments we[illegible] 1 ml platelet rich plasma plus 1 ml isotonic phosphate [illegible] 5HT for 6 min followed by centrifugation and resuspe[illegible] of unlabelled 5HT (to [bl]ock re uptake of released [illegible] bound ^{14}C 5HT during [subse]quent incubation at 37° C for 30 min was taken as a measure of spontaneous outflux

Results

1 *Immediate effects of different anions on 5HT uptake rate*

Fig 1 shows the immediate effects (as opposed to the preincubation effects — see below) of those anions which were found to be effective in replacing chloride All anions were present in the concentration of 75 mM, and the 5HT concentration was 0 4 μM, *i e* approximately corresponding to the apparent K_m for 5HT in the presence of chloride (Lingjærde 1971) The anions which could replace chloride were in decreasing order of effectiveness bromide, nitrite iodide, nitrate and formate

Orthophosphate, sulphate bicarbonate and citrate could not replace chloride neither did they show any effect in the presence of chloride Acetate alone supported uptake to a small extent but stimulated markedly in the presence of chloride (to be dealt with in detail in a subsequent paper) Fluoride alone had no effect but inhibited uptake in the presence of chloride, confirming earlier reports (see Feer 1968) A possible stimulatory effect by fluoride could thus have been counteracted by the inhibitory effect

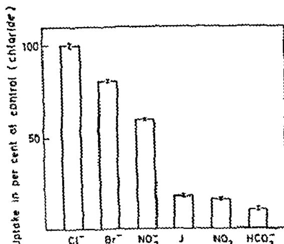


Fig 1 Effects of different anions on 5HT uptake rate The anions shown were all present in a concentration of 75 mM The mixtures were incubated for 5 min with 0 4 μM ^{14}C-5HT The mean of 3 parallel samples ± s e.m is shown in per cent of mean uptake rate in the presence of chloride

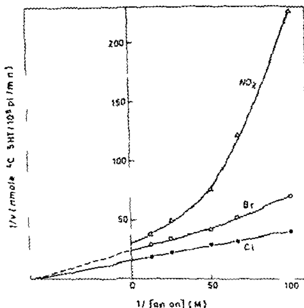


Fig 2 Double inverse plots of 5HT uptake versus concentration of chloride (•) bromide (o) or nitrite (Δ) The mixtures were incubated for 4 min with 1 0 μM ^{14}C-5HT Each point represents the mean of two parallel samples

The kinetics of the chloride, bromide, and nitrite effects are shown in Fig 2 In accordance with previous results the chloride effect adheres to simple Michaelis Menten type kinetics (Lingjærde 1971) Bromide and nitrite, on the other hand gave sigmoidal direct plots and non linear upward concave inverse plots Even though bromide and nitrite gave stronger preincubation effects than does chloride (see below), these discrepancies cannot be explained as preincubation artefacts

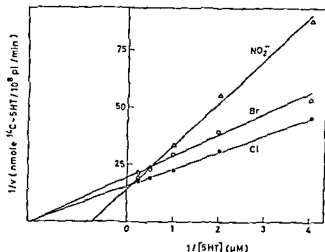


Fig 3 Double inverse plots of 5HT uptake versus concentration of 5HT, in the presence of 75 mM chloride (•), bromide (o), or nitrite (Δ) The concentration of ^{14}C 5HT was varied from 0 25 to 4 0 μM The mixtures were incubated for 4 min with ^{14}C 5HT Each point represents the mean of two parallel samples

Because of the non linearity of the inverse plots for bromide and nitrite, the kinetic parameter K_m can not be obtained for these anions However, extrapolating from the parts of the curves nearest to the ordinate in Fig 2 gives about equal intercepts on the abscissa for chloride and bromide, indicating that these anions have about the same affinity for the carrier, when present in high concentration In this experiment, $V_{max(Cl^-)}$ (i e, the uptake velocity at saturating concentrations of chloride) was higher than $V_{max(Br^-)}$ which was, again, higher than $V_{max(NO_2^-)}$ As will be seen below, however, this is only true for low concentrations of 5HT

In the experiment shown in Fig 3 platelets were incubated with varying concentrations of 5HT in the presence of 75 mM of chloride, bromide, or nitrite All plots are linear indicating that the Michaelis-Menten type kinetics as to 5HT are maintained when chloride is replaced by bromide or nitrite When chloride was replaced by bromide, the apparent K_m was unaltered, whereas $V_{max(5HT)}$ was lowered In the presence of nitrite, on the other hand, the apparent K_m is more than twice as high as in the chloride medium, and $V_{max\ 5HT)}$ is also somewhat higher Thus at low concentrations of 5HT, *the uptake is lower in a nitrite medium than in a chloride medium*, whereas saturating concentrations of 5HT give a somewhat higher uptake in the nitrite medium

Since nitrite was found to give the more marked alterations in the uptake kinetics the effect of this anion was subjected to some further studies In the experiment shown in Fig 4, platelets were incubated with four different concentrations of 5HT at four different nitrite concentrations With nitrite as the variable substrate (Fig 4 a), the inverse plots are non linear upwards concave as in Fig 2 With 5HT as the variable substrate (Fig 2 b), the inverse plots are *linear at all nitrite concentrations Increasing concentrations of nitrite give increasing* $V_{max\ 5HT)}$ and decreasing apparent K_m, indicating that the affinity of the carrier for 5HT is rather low in the

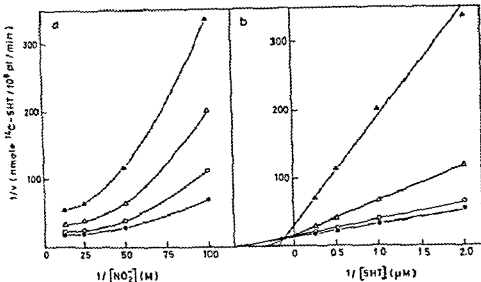


Fig 4 (a) Double inverse plots of 5HT uptake versus [NO_2] in the absence of chloride, at the following levels of [5HT] 4 μM (●), 2 μM (○), 1 μM (Δ), and 0.5 μM (▲)
(b) The same experiment, with double inverse plots of 5HT uptake versus [5HT], at the following concentrations of NO_2 80 mM (●), 40 mM (○), 20 mM (Δ), and 10 mM (▲) The mixtures were incubated for 5 min with ^{14}C-5HT Each point represents the mean of two parallel samples.

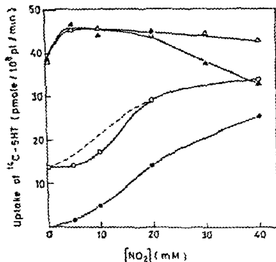


Fig 5 Uptake of 5HT versus [NO_2] at the following levels of [Cl] 0 mM (●), 10 mM (○), 60 mM (Δ) and 90 mM (▲) The mixtures were incubated for 5 min with 1.0 μM ^{14}C-5HT Each point represents the mean of two parallel samples Stippled curve is explained in the text

presence of low nitrite concentrations and increasing at higher concentrations This is in contrast to the lack of effect of chloride on apparent K_m ur (Lingjærde 1971

The effect of varying concentrations of nitrite in the presence of different concentrations of chloride is shown in Fig 5 which demonstrates the following features of

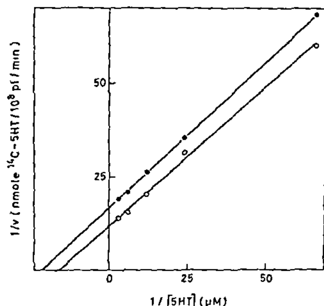


Fig 6 Double inverse plots of 5HT uptake versus [5HT] in the presence of 80 mM Cl, with (●) or without (○) 10 mM NO_2 added The mixtures were incubated with ^{14}C-5HT for 3 min Each point represents the mean of two parallel samples.

interest (1) In the presence of 10 mM chloride, the sigmoidity of the nitrite curve was even more pronounced than in the absence of chloride This was verified by making Hill plots (*i e*, log$\frac{V - V_0}{V_{max} V}$ versus log [NO_2]) of the data from the two lower curves In the absence of chloride the interaction coefficient was approximately 2 while in the presence of 10 mM chloride it was about 3 To illustrate the difference further, the theoretical curve which would have been the result if the interaction coefficient were 2 also in the presence of 10 mM chloride, is shown as a stipled curve on the Figure (It was assumed that V_{max} was the same as found for the experimental curve) In some experiments the initial parts of the upper curves *i e*, at higher concentrations of chloride, were also sigmoidal, with interaction coefficients higher than 2 (2) The effects of nitrite and chloride were partly, but not completely, additive, the maximal additional effect of nitrite being lower at higher chloride concentrations. In this experiment saturation with chloride was obtained already at 60 mM (increasing the concentration to 90 or even 120 mM did not alter the uptake rate) Nitrite still had a significant additional effect at saturating concentrations of chloride Thus, nitrite has an effect on 5HT uptake which is beyond that exerted by chloride alone (3) The maximal effect of nitrite was obtained at lower concentrations of this anion at increasing concentrations of chloride (4) At saturating concentrations of chloride, higher concentrations of nitrite had an inhibitory effect on the uptake This was more pronounced in the presence of 90 mM than 60 mM chloride

Fig 6 shows how nitrite affects the 5HT uptake kinetics in the presence of saturating concentrations of chloride The lines are almost parallel *i e*, there is about the same relative increase in V_{max} and apparent K_m

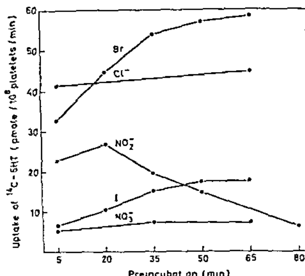


Fig 7 Effect of preincubation with different anions on 5HT uptake All anions shown were present in a concentration of 75 mM The mixtures were first preincubated for the periods of time shown on the abscissa, and thereafter incubated for 5 min with 0 5 μM ^{14}C-5HT Each point represents the mean of three parallel samples Chloride and bromide were studied in the same experiment whereas nitrite, iodide and nitrate were studied in separate experiments

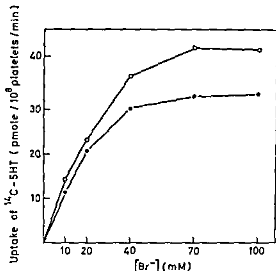
2 *Effects of preincubation*

Preincubation with high concentrations of chloride gives increased uptake rate, whereas the reverse is true for preincubation with a low chloride concentration (Lingjærde 1971) The result of preincubation with 75 mM of different anions is shown in Fig 7 *The anions differed markedly in their preincubation effects* Whereas chloride had a very small effect, bromide and iodide gave a marked increase in initial uptake rate with increasing periods of preincubation, with a levelling off after about 1 hr Nitrite gave a significant increase in uptake rate during the first 20 min, followed by a marked decrease The preincubation effect of nitrate was not significant

When platelets were preincubated with low concentrations of the different anions (*e g*, 10 mM), a gradual decrease in uptake rate was observed for all anions, as was described previously for chloride (Lingjærde 1971) However, with these low concentrations of the active anions, the uptake rates were too low to allow reliable comparisons between them

The preincubation effect of bromide was subjected to some further experiments Thus, the effect of preincubation on the kinetics of the bromide effect was studied by measuring 5HT uptake after 6 and 60 min preincubation Fig 8 shows that the main effect of preincubation was an increase of $V_{max(Br)}$, whereas $S_{0.5}$ (*i e*, the concentration of the variable substrate giving half maximal uptake rate) was practically unaltered (The direct rather than the inverse plots are shown since the latter are non linear)

The preincubation effects of chloride are probably due to changes in the intracellular concentration of this anion (Lingjærde 1971) (The extracellular concen-



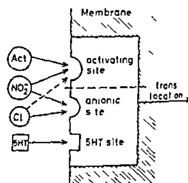


Fig 8 Fig 9

Fig 8 Effects of preincubation on the kinetics of the bromide effect The concentration of Br^- was varied from 10 to 100 mM using sulphate as an inert substitute The mixtures were preincubated without 5HT for 6 min (●) or 60 min (○), and thereafter incubated for 5 min with 0 5 μM ^{14}C 5HT Each point represents the mean of two parallel samples

Fig 9 A schematic representation of the model of the effects of anions on the 5HT uptake The carrier is only shown in the position where it is "open" outwards "Act" designates the hypothetical physiological activator For further explanation, see text

ration can be considered as unaltered during the preincubation, because the volume of the platelets make up for only about 0 25 % of the total incubation volume) To test the hypothesis that the preincubation effect of 75 mM bromide is due to the accumulation of bromide in the platelets, the following experiment was performed Platelets were first incubated with bromide or chloride, and then isolated and resuspended in a medium with only chloride In this second medium 5HT uptake was measured after 5 and 30 min preincubation Table I shows that the immediate up-

TABLE I Effect of preincubation with Br^-, followed by removal of extracellular Br^- The platelets were first incubated for 30 min in a medium with either 75 mM Br^- or 75 mM Cl^- and then isolated by centrifugation and resuspended again in a standard chloride medium In this second medium the platelets were preincubated without ^{14}C-5HT for 5 or 30 min, and thereafter incubated with 1 0 μM ^{14}C-5HT for 5 min The means of three parallel samples ± s e m are given

First preincubation in	5HT uptake (pmole/10^8 pl/min) after preincubation in second (chloride) medium for	
	5 min	30 min
bromide	21 6 ± 0 79	18 0 ± 0 23
chloride	14 9 ± 0 62	18 6 ± 2 10

take rate was about 50 % higher in the platelets previously incubated with bromide, while there was no longer any difference after preincubation for 30 min This shows that the preincubation effect of bromide is reversible and the result is also compatible with the assumption that bromide diffuses relatively slowly through the plasma membrane having a stronger intracellular effect than chloride on the 5HT uptake

3 *Effects of different anions on 5HT outflux*

The spontaneous outflux rate (see Methods) was significantly greater in the presence of bromide or nitrite (55 and 50 % of total platelet 5HT lost per 30 min respectively) than in the presence of chloride iodide nitrate, or phosphate buffer (varying between 31 and 42 %) The significance of this result is discussed below

Discussion

It has previously been argued that chloride is the physiological anion taking part in 5HT uptake *in vivo* (Lingjærde 1971) The replacement of chloride by other small univalent anions even if considered unphysiological , seems to reveal some new features of the uptake mechanism

1 *Effects of different anions on 5HT outflux* At present no explanation can be offered as to why 5HT outflux is higher in the presence of bromide and nitrite than in the presence of other anions However the more important aspect of the outflux experiments in this study, is that they show outflux to be very small in relation to influx Thus it can be calculated that the ratio of ^{14}C 5HT influx to ^{14}C 5HT outflux during 5 min incubation with ^{14}C 5HT is about 12 1 Therefore, net uptake of ^{14}C 5HT can be taken as a fairly accurate measure of influx Furthermore, the variations in outflux in the presence of different anions are too small to have any significant effect on the relationship between net uptake and influx and can therefore be disregarded as far as the rate of uptake is concerned

2 *Effects of preincubation* The different preincubation effects of the active anions can also not be satisfactorily explained It has previously been suggested that the preincubation effects of chloride are related to alterations in the intracellular concentration of this anion may be because intracellular chloride has a stimulatory effect on the retranslocation of the carrier from being open inwards to being open outwards This would be compatible with the effects of bromide as described in Fig 8 and Table I However it is not known why bromide which has less immediate effect than chloride on influx rate and which is probably less diffusible through the plasma membrane has a stronger preincubation effect (at 75 mM) than has chloride The inhibitory effect of nitrite after prolonged preincubation also awaits an explanation

The preincubation effects constitute a source of error when measuring the immediate effects of the different anions *i e* the effects which they have before any preincubation effect has been exerted (which is probably the pure extracellular effects of the anions) This error can not be completely avoided but with the minimum

temperature equilibration time of 5 min, and an incubation period of 4 or 5 min, the error is so small that the measured uptakes can still be taken as a fairly precise measure of the immediate effects of the different anions

3 *Immediate effects of ions*

3 1 *Effect of ionic size* The anions found effective in replacing chloride in the uptake mechanism were the following in decreasing order of effectiveness (referring to the experimental conditions described, *e g*, in Fig 1) bromide, nitrite, iodide nitrate, and formate Larger anions, such as orthophosphate, sulphate, bicarbonate and citrate, had no significant effect Thus, anions containing more than four atoms seem to have no appreciable effect Also within the group of active anions, there is a certain correlation between ionic size and activity, the smallest anions being more effective than the larger ones It can also be stated that no bivalent or polyvalent anions were effective in replacing chloride However, since large univalent anions are also not effective, this may be due to the large size of bivalent or polyvalent anions rather than to their charge as such

A closer analysis of the effect of ionic size is difficult, mainly because the measured over all activity (as shown in Fig 1) is the result of at least two properties which are not necessarily related, *i e*, the affinity for the carrier, and the effect on the translocation process (see Lingjærde 1971) Only in the case of bromide versus chloride is it possible to make reasonably accurate comparisons of the relevant kinetic parameters (Fig 2 and 3) Bromide in high concentration seems to have the same apparent affinity for the carrier as has chloride while its effectiveness on the uptake nce bound to the carrier) is only about 60 % of that of chloride Furthermore the ffinity of 5HT for the carrier is not significantly altered when chloride is replaced by bromide (Fig 3) Since bromide is a larger anion than chloride it may tentatively be concluded that the larger the anion, the less effective it is on the translocation process Furthermore is seems reasonable to assume that the affinity of an anion for the carrier is also roughly inversely proportional to its size If for example anions like orthophosphate or bicarbonate could be bound to the anionic site of the carrier they would be expected to exert a competitive inhibitory effect versus chloride, which they do not

3 2 *The effects of nitrite* warrent special consideration, since they are markedly different from those of chloride

The simple model of the effect of chloride which was presented earlier (Lingjærde 1971) showed the following main features The carrier was supposed to have one site for chloride the 'anionic site and one site for 5HT Furthermore it was assumed that binding of chloride to the anionic site did not affect the affinity of the carrier for 5HT, but was necessary for the translocation of the carrier from being "open outwards to being open inwards However, this simple model can not explain the effects of nitrite

Since nitrite (like other small anions such as bromide iodide and nitrate) can replace chloride in the over all uptake process it is reasonable to postulate that it can

react with the anionic site of the carrier in the same way as does cloride However, nitrite has a stimulatory effect on the uptake which goes beyond that exerted by saturating concentrations of chloride (Fig 5 and 6) We shall call this the "specific activating effect" of nitrite, and postulate that it is due to an effect on a hypothetical "activating site on the platelet membrane, probably on the carrier itself

A possible explanation for the sigmoidal curve shown by nitrite, is that the carrier is an allosteric protein, consisting of two or more identical protomers (Monod *et al* 1965), each with an anionic site, a 5HT site, and an activating site, and able to exist in at least two different configurations (each being able to be oriented either outwards or inwards), with different transport activities However, some experimental findings do not easily fit into an allosteric model Firstly, chloride or 5HT never give sigmoidal uptake plots, whether an activator is present or not. Secondly, the kinetics of the chloride and 5HT effects indicate that each of these compounds is bound only once to the functional carrier unit (Lingjærde 1971)

A simpler model, which seems to fit the experimental data better (although it cannot easily explain all data), is the one illustrated in Fig 9 This model differs from the previously proposed model only by inclusion of the above mentioned 'activating site' besides the anionic and 5HT sites (It should be noted that this model, like the previous one, represents an attempt only to explain the effects of anions, not the effects of, *e g*, sodium (Lingjærde 1969)) Nitrite and other specific activators can transform the carrier from a "non activated' (but still active) to an 'activated' state, which is assumed to increase the affinity of the anionic site for chloride (or other small anions acting at the anionic site) In the absence of a specific activator, the carrier is always in the non activated state, and will behave according to the former model (Lingjærde 1971) The effect of a specific activator on the affinity of the anionic site is in accordance with the positive cooperativity (i e, the sigmoidal uptake curve) shown by nitrite Increasing concentrations of nitrite transform the carrier progressively into the activated state by an effect on the activating site, and this in turn enhances the binding and thereby the effect, of nitrite in the anionic site (It is more difficult to show directly that nitrite increase the affinity of the anionic site for chloride, because of the complex interactions of these anions However, in a forthcoming paper it will be shown that another specific activator, acetate, which does not interact with the anionic site, has a marked effect on the affinity for chloride)

The effect of nitrite on the 5HT affinity is more complex At low concentrations of nitrite, the apparent affinity of 5HT is very low, but it increases with increasing concentrations of nitrite However, even with 75 mM nitrite, the apparent affinity of 5HT is much lower than in a pure chloride medium (Fig 3) This effect of nitrite on 5HT affinity is in contrast to the lack of effect of chloride concentration on 5HT affinity (Lingjærde 1971) It may be related to the fact that nitrite is a much larger anion than chloride, thus exerting some unphysiological effects on the anionic site, and indirectly, also on the 5HT site

The experiment shown in Fig 5 reveals several interesting interactions

chloride and nitrite, some of which can not be satisfactorily explained at present It is thus seen that higher concentrations of nitrite have an inhibitory effect when present together with a high chloride concentration, and the effect is greater at 90 mM chloride than at 60 mM chloride, although both these chloride concentrations were saturating in this experiment It is also seen from the Figure that 10 mM chloride increases the sigmoidity of the nitrite curve i e, it reduces the effect of lower concentrations of nitrite One possible explanation is that chloride has a certain affinity for the activating site, without being able to exert a specific activating effect, and thus acting as a competive inhibitor of nitrite at this site

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Effects of Pilocarpine on L-Dopa Turnover in the Exocrine Rat Pancreas

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Abstract

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The following abbreviations are used in this article DOPA = 3 4-dihydroxyphenylalanine, DA = dopamine 5 HTP = 5-hydroxytryptophan 5 HT = 5 hydroxytryptamine, MAO = monoamine oxidase COMT = catechol O-methyl transferase
The turnover of L-DOPA was microscopically and chemically studied in rats after pretreatment with pilocarpine alone or in combination with nialamide Whenever pilocarpine was given there was an almost complete extrusion of fluorescent (i e dopamine containing) zymogen granules from the exocrine cells In such cases, specific fluorescence frequently ap-

The exocrine pancreatic cells have been shown to possess a special capacity for the uptake and handling of DOPA and 5 HTP (Rosell, Sedvall and Ullberg 1963, Ritzen, Hammarström and Ullberg 1965, Gershon and Ross 1966 a and b, Alm, Ehinger and Falck 1969, Alm 1969) From these amino acids, extensive quantities of the respective amines, DA or 5 HT, are formed intracellularly The amines are to some extent probably taken up and stored in the zymogen granules, this uptake-storage mechanism" being affected by some psychotropic drugs (Alm *et al* 1971 a) A large amount of the DA formed probably located outside the zymogen granules seems to be degraded to deaminated metabolites (Alm 1971)

The aim of the present investigation was to find out whether the pancreatic turnover of L DOPA is influenced by the changes in the pancreatic secretion that can be induced by the administration of pilocarpine (see reviews by Hansson 1959 Hermodson 1965, Nevalainen 1970)

Materials and Methods

Albino rats of both sexes (Sprague Dawley, 180—250 g b wt , Anticimex, Sweden) were used. All the animals were kept in similar conditions and given standard pellets (Teknosan Sweden) and water *ad libitum*

Experimental procedure The experiments were performed between 10 a m and 3 p m All injections of drugs were made i v as previously described (Alm *et al* 1969) At different times after the injections of L DOPA (see below and Table I) the animals were killed by a blow

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when present in different mutual concentrations It was found that when one of the substances (DOPA or DA) was present in a very high concentration the other substance could be falsely scored or scored as absent In such cases, paper chromatography was performed on extracts from pooled pancreatic glands If it was possible to demonstrate the presence of the substance in question this was denoted as chromatographically detectable (*cf* Table I as no meaningful estimate of the concentration could be made (*cf* Alm *et al* 1971 a) The technique described was used in this work when DA occurred in a very high concentration compared to DOPA

Student s *t* test was used to test the significance of differences of concentrations (Documenta Geigy 1962) at observations after 10 or 40 min only, as for morphological reasons these times represent different phases in the pancreatic L DOPA turnover (*cf* Alm *et al* 1971 a)

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Drugs L DOPA (Sigma Chem Corp) dissolved in 0 9 % saline was injected i v (40 mg/kg) Nialamide solution (Niamid Pfizer Ltd) was injected i v (100 mg/kg) 3 h before the injection of L-DOPA for the inhibition of MAO Pilocarpine hydrochloride dissolved in 0 9 % saline was injected both i v (40 mg/kg) and i m (40 mg/kg) 3 h before the injection of L-DOPA In some expts nialamide was given simultaneously with pilocarpine as described below

Microscopy Fluorescence microscopy was performed according to the method of Falck and Hillarp the fluorescence picture was judged according to the intensity of the fluorescence and localization (*cf* Alm 1969 Alm *et al* 1971 a) When it could not be localized to any cellular structure it was denoted as diffuse when it was confined to zymogen granules, as identified in the phase contrast microscope it was characterized as granular The intensity was arbitrarily denoted in four different grades as weak moderate, strong and very strong It should be emphasized that the fluorescence intensity is not always strictly proportional to the amine concentration (Ritzén 1967) but that visual estimates can none the less be used with a fair degree of confidence (Olsson *et al* 1968)

Results

Histochemical observations

The fluorescence picture in animals given L DOPA alone or in combination with inhibitors for DOPA decarboxylase COMT and MAO, has been described in detail by Alm (1971 and Alm *et al* (1971 a) 10 min after the administration of L-DOPA alone, granular fluorescence appeared the most basally located zymogen granules demonstrating a distinctly stronger fluorescence intensity than those lying more apically From 20 min onwards the different zymogen granules did not show differ

Fig 1 a. Rat pancreas 40 min after the injection of L DOPA (40 mg/kg) Specific fluorescence occurs in coarse granules in the apical parts of the exocrine cells Fluorescence micrograph 438 ×

Fig 1 b Phase-contrast micrograph areas and magnification same as in Fig 1 a. Note the close correspondance between the coarse fluorescent granules (see above) and the zymogen granules.

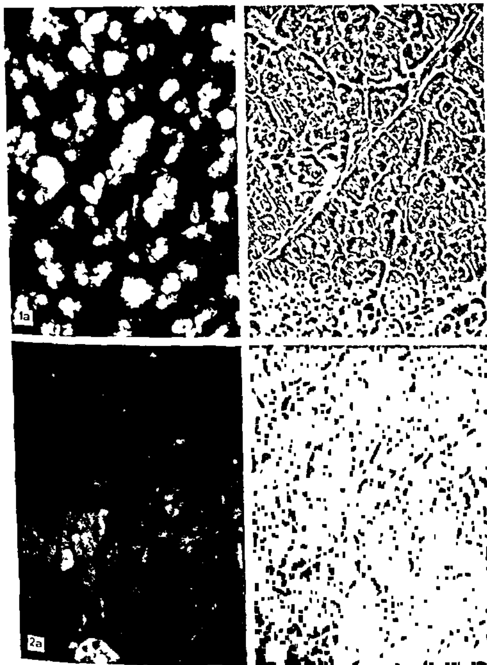


Fig 2 a Exocrine acini 40 min after the administration of L DOPA in a pilocarpine pretreated rat Almost no specific fluorescence is seen (compared with Fig 1 a) Part of an endocrine islet is seen to the left Fluorescence micrograph 438 ×

Fig 2 b Phase-contrast micrograph area and magnification same as in Fig 2 a The exocrine acini are almost devoid of zymogen granules (compare with Fig 1 b)

TABLE I

		Normal animals	
		DOPA	DA
L-DOPA	10	6 40±0 79 (20)*	43 4 ±3 76 (20)*
	20	5 30±1 21 (20)*	17 7 ±1 94 (20)*
	30	1 63±0 28 (16)*	10 4 ±0 73 (16)*
	40	0 56±0 13 (16)*	9 30±1 14 (16)*
	60	0 37±0 09 (8)*	2 88±0 45 (8)*
	90	0 46±0 16 (8)*	1 99±0 21 (8)*
Nialamid +L-DOPA	10	cd**	101 ±5 08 (14)**
	20	cd**	84 0 ±8 80 (9)**
	30	cd**	76 6 ±5 70 (8)**
	40		
	60	1 14±0 55 (8)**	56 6 ±5 53 (8)**
	90		
	120	1 72±0 19 (6)*	13 5 ±1 56 (6)*

* Figures taken from Alm (1971) ** Figures taken from Alm *et al* (1971 a)
cd = chromatographically detectable, see Materials and Methods The DOPA and DA values are expressed as μg/g wet tissue (M±S E) The figures within brackets incidate number of animals

ences in intensity Very strong granular fluorescence occurred up to 40 min after the L-DOPA injection (Fig 1 a and b) and thereafter could be denoted moderate to strong The diffuse fluorescence outside the zymogen granules was moderate to strong after 10 and 20 min weak after 30 min and thereafter apparently absent

Inhibition of MAO before the L-DOPA administration resulted in both a strong diffuse fluorescence all over the cells and a very strong granular fluorescence 10 to 30 min after the L-DOPA administration In the 60 min expts, seemingly all zymogen granules emitted a very strong fluorescence, the diffuse fluorescence being weak to moderate

Administration of pilocarpine, alone or together with nialamide before the administration of L-DOPA resulted in an almost total extrusion of the zymogen granules (Fig 2 b) In few acini a very restricted number of zymogen granules occurred in the most apical cellular parts Frequently, the acini lying close to the islets of Langerhans (peri insular acini) did not appreciably seem to have lost any zymogen granules a result of the special behavior of the peri insular acini (cf Sergeyeva 1938) Moreover at all times tested, the granular fluorescence was enormously reduced and could only be found in the exocrine cells still containing zymogen granules (Fig 2 a) thus further emphasizing the localization of granular fluorescence to zymogen granules Finally specific fluorescence was frequently seen in the acinar lumina and the secretory ducts 10 and 20 min after L-DOPA administration, this was in contrast to the picture seen in animals not pretreated with pilocarpine.

When only pilocarpine was given before the L-DOPA injection there also appeared a diffuse fluorescence After 10 min, it was strong after 20 and 30 min,

Pilocarpine pretreatment (uncorrected values)		Pilocarpine pretreatment (values corrected for pancreatic losses of weight)	
DOPA	DA	DOPA	DA
21 8 ±3 38 (12)	60 9 ±4 48 (12)	16 4 ±2 54 (12)	45 7 ±3 36 (12)
1 75±0 17 (10)	12 3 ±2 44 (10)	1 31±0 13 (10)	9 23±1 83 (10)
0 73±0 15 (11)	4 86±0 59 (11)	0 55±0 11 (11)	3 65±0 44 (11)
0 28±0 04 (11)	2 65±0 22 (11)	0 21±0 03 (11)	1 99±0 17 (11)
0 23±0 03 (8)	1 49±0 15 (8)	0 17±0 02 (8)	1 12±0 11 (8)
0 14±0 03 (8)	0 54±0 08 (8)	0 11±0 02 (8)	0 41±0 06 (8)
cd	102 ±5 78 (8)	cd	76 2 ±4 34 (8)
cd	117 ±8 14 (8)	cd	87 8 ±6 11 (8)
0 48±0 19 (11)	55.5 ±9 03 (11)	0 36±0 14 (11)	41 6 ±6 77 (11)
0 13±0 05 (13)	26 3 ±5 92 (13)	0 10±0 04 (13)	19 7 ±4 44 (13)

was very weak, slightly exceeding the pancreatic autoflourescence From 40 min onwards, the exocrine cells were seemingly devoid of specific fluorescence

Pretreatment with pilocarpine and nialamide simultaneously before the L-DOPA administration resulted after 10 min in a strong diffuse fluorescence, which there after remained moderate

Chemical determinations

The chemical estimates are summarized in Table I The animals given L-DOPA or nialamide and L-DOPA are referred to as normal animals in this work and the corresponding values of DOPA and DA are taken from Alm (1971) or Alm *et al* (1971 a)

In accordance with observations by Farber and Sidransky (1956) it was found in a pilot study (20 rats) that, after pilocarpine administered in the doses described in this work the weight of the whole rat pancreatic gland clearly decreased when correlated to the b w of the rat that was unchanged (mg wet pancreatic tissue per 100 g b w) The decrease of the pancreatic weight was approximately the same (25 0 % = 'average loss of weight , see below) at three as well as five hours after the pilocarpine administration *i e* the time period during which the animals were sacrificed This was allowed for in the calculation of the DOPA and DA values in pilocarpine pretreated animals when compared with animals that had not received pilocarpine in order to get more comparable results [1]

[1] The chemical determinations are calculated as a/b where a = total amount of the substance found (expressed in μg) and b = wet pancreatic weight (expressed in g) Correction of concentrations for pancreatic weight losses after pilocarpine pretreatment (see above) is calculated according to the following formula

$$\frac{a \cdot (100 - \text{"average loss of weight"})}{b \cdot 100}$$

The numerical value of 'average loss of weight when used in the formula is 25 0 (see above)

Pretreatment with pilocarpine changed the course of the pancreatic DOPA and DA turnover compared with normal animals The DOPA level was unchanged (P > 0 05) but the DA level was clearly higher (P < 0 01) 10 min after the L-DOPA administration They then decreased more strongly than in normal animals From 20 min onwards both the DOPA and DA levels in pilocarpine pretreated animals were lower than corresponding DOPA and DA levels in normal animals

Pretreatment with both malamide and pilocarpine resulted in lower amounts of DA than after pretreatment with only malamide 10 min after the L-DOPA administration (P < 0 01) Moreover, in the first group the DA level had not changed after 40 min (P > 0 05) From this time point the pancreatic DA level diminished to about 20 μg/g at 90 min In the animals not pretreated with pilocarpine the DA level consistently decreased from about 101 μg/g (at 10 min) to about 13 μg/g (at 120 min) In both the groups the DOPA level was low

Discussion

Compared with the endocrine islet cells, the adrenergic nerves the small intensely fluorescent catecholamine containing cells (SIF-cells) and very few enterochromaffin cells occurring in the largest pancreatic ducts the exocrine cells constitute a dominant part of the total number of cells that can take up and decarboxylate L-DOPA and L 5 HTP to their corresponding amines (cf Cegrell 1968, Alm et al 1969 Moreover only very small amounts of noradrenaline, and probably DA can be found in the rat pancreas not exposed to drugs (Alm, Liedberg and Owman 1971 d Therefore as presumed by Alm et al (1971 a) the chemical estimates reflect to a negligible extent changes in the DOPA and DA concentrations of non exocrine cells In this connexion it should be noted that pilot expts revealed no difference in the pancreatic L-DOPA turnover between male and female rats (cf Alm 1971)

The discharge of enzyme proteins from exocrine pancreatic cells can be stimulated by two principally different mechanisms either hormonally by the influence of pancreozymin or nervously by vagal stimulation (cholinergic stimulation) (cf Grossman 1962) Cholinergic drugs mimic vagal stimulation Among these pilocarpine has been widely used and some of the effects on the exocrine pancreatic cells have been investigated both morphologically and chemically (cf Farber and Sidransky 1956 Nevalainen 1970 In this connexion it must be emphasized that there has been much discussion whether or not pilocarpine evokes an accelerated protein synthesis in the rat pancreas concomitant with the increased well established enzyme secretion (cf Kramer and Poort 1968 Morisset and Webster 1971)

In cats and dogs the pancreatic secretion is very slight during starvation (Lin and Grossman 1956 Hansson 1959) This is in contrast to rodents such as guinea pigs mice, and rats described as having a continuous secretion that is not or only very slightly affected by starvation (Love 1956 Hansson 1959) Consequently in this work experiments were performed only on rats with a stimulated pancreatic secretion

In this study, an almost total extrusion of zymogen granules from the exocrine

cells was found when pilocarpine was given (*cf* Nevalainen 1970) Moreover, whenever pilocarpine was administered before the L-DOPA administration, almost no granular fluorescence occurred in the exocrine cells except in the peri insular acini This clearly demonstrates that the granular fluorescence previously observed in the non pilocarpine pretreated animals is confined to the zymogen granules (*cf* Alm *et al* 1969, Alm *et al* 1971 a) The occurrence of very few zymogen granules in a few exocrine cells is perhaps due to the fact that the exocrine cells work asynchronously of each other concerning enzyme synthesis and secretion (*cf* Sjöstrand 1962)

Except for the 10 min observation time, pretreatment with pilocarpine before the administration of L DOPA produced lower DOPA and DA values at the times studied compared with normal animals Correspondingly weak or no specific fluorescence appeared in the histochemical analyses It perhaps seems somewhat odd that despite pancreatic concentrations of DA in the order of 4—10 μg/g (*cf* Table I), almost no specific fluorescence could be demonstrated The reason for this seems to be that after pilocarpine pretreatment when no zymogen granules occur, the fluorophore is evenly distributed throughout the cytoplasm, in contrast to non pilocarpine pretreated animals, when it is concentrated to the zymogen granules, which in the form of small spots can be more easily localized The argument is further strengthened by the observations that pretreatment with pilocarpine and malamide resulted in a diffuse fluorescence evenly spread over the cells Although a high pancreatic DA level was recorded, the diffuse fluorescence did not reach a higher than moderate intensity This was distinctly lower than that of exocrine cells 40 min after the administration of L-DOPA alone, which only showed granular fluorescence and had a much lower DA content (about 9 μg/g) The observations are good examples of the errors inherent in subjective estimates in the microscope and emphasize the need for controls by chemical determinations of concentrations

10 min after the L-DOPA administration pretreatment with pilocarpine resulted in higher DOPA but unchanged DA levels compared with normal animals These observations suggest that pilocarpine produces an initial increase in the pancreatic uptake of L-DOPA. This is interesting compared with findings by Hansson (1959), demonstrating increased concentrations of methionine in the acid soluble (non protein) fraction of the pancreas after pilocarpine pretreatment It is noteworthy that in this respect there seems to be no difference between the uptake of the protein forming amino acid (methionine) and L DOPA, not supposed to take part in the synthesis of proteins

The DOPA and DA concentrations in pilocarpine pretreated animals decreased more rapidly than in non pretreated animals Further, comparison between the rate of the DA elimination after pretreatment with pilocarpine and malamide (from the observations at 40 min and onwards) and malamide only (the whole test period) shows that the DA elimination is faster in the first group as larger quantities of DA disappear during a shorter time This seems to indicate that the accelerated pancreatic elimination of DOPA and/or DA by pilocarpine could be due to the stimulated pancreatic secretion rather than to an increase in catabolism

In animals given L-DOPA only, no specific fluorescence was observed in the acinar lumina and the secretory ducts However, after pretreatment with pilocarpine such a fluorescence was clearly demonstrable It has been proposed above that specific fluorescence in the pancreas is more easily discovered when it is confined to the zymogen granules An increased protein content of the pancreatic juice in the acinar lumina and secretory ducts after pilocarpine pretreatment could to some extent explain the occurrence of specific fluorescence in these parts of the secretory system with respect to the known influence of proteins present for the outcome of the histochemical fluorescence reaction (*cf* Falck *et al* 1962, Corrodi and Hillarp 1963 1964) In this connexion it should also be mentioned that such a specific fluorescence was seen after the administration of very high amounts of L-DOPA (500 mg/kg i p Alm unpublished observation), suggesting that increased concentrations of fluorogenic material in the acinar lumina and secretory ducts can also be responsible for the appearance of specific fluorescence in these glandular parts

In studies with electron microscopic autoradiography, it has been shown that at certain times after the administration of tritiated DOPA and 5 HTP, silver grains (at this site probably representing the corresponding amines) predominately appear over the zymogen granules, but that later most of the radioactivity can be localized to the acinar lumina and the secretory ducts (Alm, Ekholm and Ericson 1971 b) Analyses of pancreatic juice have demonstrated the presence of radioactive substances after the administration of ^{14}C-L-DOPA (Alm, Ekholm and Ericson 1971 c) These findings favour the suggestion that the labelled material which accumulated to the zymogen granules is released into the acinar lumina concomitantly with the secretion of zymogen granules However, after pilocarpine pretreatment, although almost no zymogen granules appeared in the exocrine cells specific fluorescence occurred in the acinar lumina and secretory ducts Thus, it cannot be excluded that after L-DOPA administration the exocrine cells can discharge DOPA or DOPA metabolites into the acinar lumina independently of the release of zymogen granules

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From the Department of Clinical Physiology, Karolinska sjukhuset and the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Discharge Patterns in Motor Nerve Fibres during Voluntary Effort in Man

By

Ulla Freyschuss and Evert Knutsson

Studies of discharges in motor nerves during voluntary muscle contractions (Hagbarth and Vallbo 1968) are hampered by the difficulty to discriminate impulses in motor nerves from those of sensory origin The technique used in a previous study of autonomic responses to intended contractions in muscle groups with a blocked neuromuscular transmission (Freyschuss 1970) seems to constitute a basis for the discrimination of true motor nerve discharges since activation of motoneurones may then be obtained during complete elimination of movements and tension changes Hence, the nerve discharges in peripheral nerves were studied during effort to contract muscles which were blocked at the motor end plate by a depolarizing agent

In 13 healthy volunteers nerve impulses were recorded with commercial microelectrodes inserted percutaneously into the median or ulnar nerves just above the wrist (Knutsson and Widen 1967) In order to facilitate the location of the electrode tip close to a bundle of motor fibres an electric pulse of 2—6 V was passed through the electrode which was moved until a twitch response was obtained When a desired localization had been attained the muscle groups, which were activated during handgrip, were paralyzed through intra arterial administration of succinylcholine SCh, (Celocurin Klorid® 5—7 mg) A complete neuromuscular blockade was verified according to the criteria described by Freyschuss (1970) The nerve impulses which were repeatedly elicited at intentions to perform a handgrip were regarded as motor nerve discharges

In 6 of the subjects such impulses were recorded A typical example is given in Fig 1 This subject was trained to perform short lasting (8 s) handgrips of various intensities During effort subjectively corresponding to less than half of the maximal no nerve impulses were obtained The discharges in Fig 1 A were evoked during an intended contraction roughly half of the maximal intensity while 1 B shows the activity during strong effort Repetitive discharges in two fibres were seen The frequency was highest during the initial period of intention whereafter it successively decreased and ceased prior to the end of the voluntary effort Another prominent feature was the relatively low frequency (30/s) even during maximal effort



Fig 1 Motor nerve discharges in the median nerve during intended activation of muscles with a blocked neuromuscular transmission *In A the volitional effort is about half of maximal in B nearly maximal* Lower beams indicate the period of intended contraction Horizontal bar, 1 s, vertical bar 1 mV

Since SCh gives rise to an increased outflow from the muscle spindles (Granit, Skoglund and Thesleff 1953) excitability changes in the motoneurone might be expected Whether inhibitory or facilitatory effects will dominate cannot be evaluated, since the peripheral effects of the drug are not selective, spindles in both synergists and antagonists being activated Transient random discharges in motor units during the first seconds after SCh administration would indicate an excitatory drive, but these discharges ceased as the block became complete and sustained resting discharges of the motor fibres were never seen Hence, a drug induced inhibition of the motoneurones cannot be excluded but other mechanisms might also explain the relatively low degree of activity in the motor fibres even during maximal effort to contract Thus a maintenance of voluntary contraction might require a concomitant excitation of the fusimotor system (Eldred, Granit and Merton 1953) which was inoperative by the neuromuscular blockade of intrafusal fibres

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From the Department of Physiology, University of Goteborg Sweden

The Effect of Intense Treatment with Hypotensive Drugs on Structural Design of the Resistance Vessels in Spontaneously Hypertensive Rats

By

Bjorn Folkow, Margareta Hallbäck, Yen Lundgren and Lilian Weiss

Hemodynamic studies of regional vascular beds in human essential hypertension suggest that the increased flow resistance is to a great extent caused by a structural adaptation of the systemic resistance vessels (Folkow *et al* 1958 Sivertsson 1970 This structural adaptation appears to be an exponent of a normal vascular response to changes in pressure load and implies an increased wall/lumen ratio mainly due to a media hypertrophy which enhances resistance even during maximal dilatation by encroaching upon the lumen Many histological studies of the resistance vessels have demonstrated a considerable media hypertrophy in human hypertension particularly convincingly that by Furuyama (1962), though the hemodynamic consequences of this hypertrophy have hardly at all been considered previously

Recent hemodynamic studies of the spontaneously hypertensive rat (SHR) (Folkow *et al* 1970) have confirmed the mentioned findings in man For example the entire systemic vascular bed of SHR and their hindquarters exhibits a raised flow resistance even during maximal dilatation as compared to normal control rats (NCR) Further the resistance vessels of the NCR SHR hindquarters show the same threshold sensitivity to noradrenaline (NA) but when exposed to supra threshold NA doses the SHR vessels display an increased steepness of the dose response curve and also an increased maximal pressor response reflecting an enhanced contractile strength All these characteristics of the SHR resistance curves are to be expected if the resistance vessels of the SHR and NCR differ mainly in terms of the bulk of media tissue The structurally enhanced wall/lumen ratio in SHR appears to be marked enough to be able to cause largely the entire rise of resistance during rest, without necessitating any enhanced smooth muscle tone

The question arises how procedures aiming at reducing the pressure load for a substantial period of time will affect the characteristic changes of the SHR resistance curve, which thus seem to reflect hemodynamically important adaptations of vascular architecture Suitable arterial obstructions reducing regional arterial pressure to about 50—70 % of the initial level were performed in the cat hindlimb (Folkow

and Sivertsson 1968) and in the hindquarters of young NCR SHR rats (Folkow *et al* 1971 a) Within few weeks the resistance curves were changed in the direction of that of low pressure circuits , with obvious signs of a reduced wall/lumen ratio and of considerable luminal widening — Further, immunosympathectomy in new born SHR litters prevented the development of high blood pressure and their resistance curves revealed a considerable normalization when compared to un treated NCR and SHR (Folkow *et al* 1971 b)

However in these studies of SHR quite young animals were used and the question arises what will happen if blood pressure is for some period of time kept pharma cologically reduced in old SHR with severe and longstanding hypertension To in vestigate this problem a pilot study was performed on SHR of more than one year of age and with average blood pressures between 225 and 250 mm Hg as measured by the tail plethysmographic method They were given hydralazine and guanethidine [1] administered in the drinking water (3 5 and 15 mg/100 ml respectively) after initial intraperitoneal injections of guanethidine In this way blood pressure was reduced to about 100—120 mm Hg within 4 weeks and was kept at this normalized level for another 8 weeks after which all treatment was interrupted for 1 week in half of the group to get some indication of how rapidly and to what extent blood pressure would rise again During this week blood pressure rose very little in the untreated animals from a mean of 105 up to only about 125 mm Hg

In order to explore how the pharmacologically induced normalization of the blood pressure level had affected the resistance curves the hindquarters of the treated SHR were artificially perfused in parallel with those of untreated matched NCR us ng the same procedures for constant flow perfusion as earlier described in detail (Folkow *et al* 1970)

During maximal vasodilatation there was no significant difference in resistance between the treated SHR and untreated NCR Thus the repeatedly noticed con siderable increase of resistance at maximal dilatation in hypertensive rats was abolished as a result of the pharmacologically induced period of pressure normaliza tion Also in other respects the resistance curves of the treated SHR exhibited a con s derable but not complete normalization compared with those of untreated SHR Thus the maximal pressor response was well below that of young SHR where arterial pressure had by far not reached the very high levels present in the old SHR before the hypotensive treatment was started

This preliminary study thus suggests that a rapid and considerable regression of the hypertrophic vascular adaptation to high pressure takes place once the pressure load is kept reduced to about normal levels for only 8 weeks (perhaps less even in old animals The fact that these rats for long time had been severely hypertensive makes it likely that secondary changes of degenerative lesional nature see Okamoto 1969 may have been added to the media hypertrophy In any case the main but probably not only background of the observed rapid regression is likely to be a considerable

[1] We are ndebted to Ciba Geigy Basel Sw tzerland for generous gifts of these drugs (Apresolin® and Ismelin®)

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From the Department of Physiology, University of Göteborg, Sweden

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By

BJÖRN FOLKOW, MARGARETA HALLBÄCK, YEN LUNDGREN and LILIAN WEISS

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The question arises how procedures aiming at reducing the pressure load for a substantial period of time will affect the characteristic changes of the SHR resistance curve, which thus seem to reflect hemodynamically important adaptations of vascular architecture. Suitable arterial obstructions, reducing regional arterial pressure to about 50—70 % of the initial level, were performed in the rat hindlimb (Folkow

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From the Departments of Histology and Physiology, University of Goteborg Sweden

The Distribution of Na in Intestinal Villi during Absorption of Sodium Chloride

By

HENGO HALJAMÄE, MATS JODAL, OVE LUNDGREN and JOAR SVANVIK

The concentration of urine in mammals is accomplished by means of a countercurrent multiplier in the renal papillae During its passage through the distal tubuli and the collecting ducts, the urine is being equilibrated with the surrounding hyper osmolar interstitial contents, produced by the renal countercurrent multiplier

It was recently demonstrated (see *e g* Lundgren 1967) that a countercurrent exchange also exists in the mucosa of the small intestine between the nonbranching central arteriole' in a villus and the surrounding dense subepithelial capillary network The intestinal countercurrent mechanism was demonstrated to be highly efficient for easily diffusible lipid soluble solutes, such as oxygen, krypton and antipyrine It was considered to be of interest to investigate whether the counter current mechanism was also operating during the absorption of water soluble solutes If so, one would in analogy with the findings in the renal medulla, expect to find a concentration gradient of the hydrophilic solute along the length of the villi the highest concentration being at their tips Furthermore the magnitude of such a concentration gradient should be dependent of the efficiency of the countercurrent exchanger

Methods

The experiments were performed on cats deprived of food for at least 24 h and anesthetized with chloralose (50 mg/kg b w) Arterial blood pressure was measured from the left femoral artery by a mercury manometer Total venous outflow from 3—5 sympathetically denervated jejunal segments weighing altogether 25—40 g was recorded by an optical drop recorder unit operating an ordinate writer

Each jejunal segment was cut open along the antimesenteric border and firmly mounted flat on a metal frame The mucosal surface was continuously flushed with an isotonic solution of sodium chloride at 37° C for 15—20 min The flushing was performed by means of an infusion pump at a rate of 1—2 ml/min Immediately after stopping the infusion of sodium chloride the segment was rinsed with approximately 40 ml of a bodywarm isotonic sucrose solution to remove the saline from the mucosa. The intestinal segment mounted on its metal frame was then swiftly extirpated and momentarily frozen in liquid nitrogen The sucrose flushing and f [illegible] in 10 or 15 μm thick sl [illegible] water After at least 2 [illegible] as pipetted from the supernatant The sodium concentration of this solution was determined by means of the technique described by Haljamae and Larsson (1968) The protein content of the remaining 300 μl was estimated by the Lowry method (Lowry *et al* 1951)

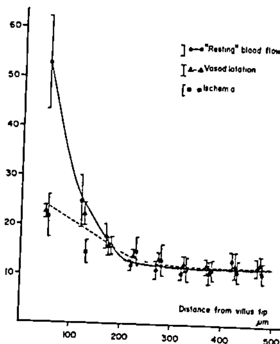


Fig 1 The distribution of sodium intestinal villi of the cat during "resting" blood flow, during maximal vasodilatation and during ischemia Lines drawn by inspection Bars indicate S E (n=5)

The present study was performed during three different experimental situations 1 during "resting" intestinal blood flow, 2 during maximal vasodilatation (around 225 ml/min× 100 g) induced by a close intraarterial infusion of isopropylnoradrenaline, 3 during total ischemia accomplished by clamping the vascular supply to the segment with a pair of Peans forceps In the lastmentioned type of experiment the saline solution was continuously equilibrated with pure oxygen

Results and Comments

It is evident from the Fig 1 that, during the absorption of NaCl the amount of sodium per unit weight protein in the tips of intestinal villi is significantly higher during "resting" blood flow than during intense vasodilatation This observation may be explained by assuming that the absorption of sodium is blood flow limited However, it is generally believed that this is not the case (Davenport 1966, p 170) and mucosal blood flow has been estimated to be rather high even during "resting" conditions (40—70 ml/min × 100 g mucosa Lundgren 1967, Kampp and Lundgren 1968) The low villous concentration of sodium found during ischemia (see figure also supports the conclusion that sodium transport across the intestinal epithelium is not blood flow limited

There exists, as pointed out in the introduction, strong evidence for the existence of a countercurrent exchange mechanism in the intestinal mucosa of the cat Such a mechanism could easily explain the results of the present study, since vasodilatation and ischemia reduce, or completely abolish, the efficiency of the intestinal counter

current multiplier and, hence, the concentration gradient along the villi It is, accordingly proposed that such a countercurrent multiplier for sodium exists in the intestinal mucosa of the cat during absorption of sodium chloride Further investigations to test this hypothesis are in progress

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

The Presence of a Sulphomucopolysaccharide-Protein Complex in Adrenal Medullary Cell Granules

By

Gilles Fillion*, Radomir Nosál** and Borje Uvnäs

The occurrence of ATP together with biogenic amines in the storage vesicles of adrenals, adrenergic neurons, thrombocytes etc, has led to the generally accepted hypothesis that the amines are stored in ATP-amine complexes Similarly it was supposed that, in mast cell granules, histamine (Hi) and 5-hydroxytryptamine (5-HT) are stored in complexes with heparin

However, recent experimental evidence has suggested that the Hi and 5 HT in mast cells is electrostatically attached to a granule heparin-protein complex with the properties of a weak cation exchange material, protein carboxyl groups acting as the amine binding sites (Uvnas, Åborg and Bergendorff 1970, Bergendorff and Uvnas 1971) Later a sulphomucopolysaccharide-(SMPS)-protein complex with the ability to bind 5-HT and Hi was found in rat thrombocyte granules (Åborg and Uvnas 1971) The mast cell granules were shown to bind not only Hi and 5-HT but also other biogenic amines, such as adrenaline (A), noradrenaline (NA) and dopamine (DA) This observation prompted us to study the possible occurrence of SMPS-protein complexes in other biogenic amine stores In the present paper some preliminary data from studies on adrenal medullary cell granules will be given

Methods

[illegible] labelled with ^{35}S (Nosál Slovak [illegible] le SMPS in the adrenal medullary [illegible] i p (8 and 20—25 mCi respectively) [illegible] h before the animals were sacrificed To label the amines contained in the vesicles 200 μCi of ^{3}H DL-NA was given i v 1 h before sacrifice

The adrenal medullas were excised and rinsed in ice cold saline homogenized in isotonic sucrose and centrifuged through a continuous sucrose density gradient (Potter and Axelrod 1963) The centrifuge tubes were punctured from below and fractions of 3—5 drops were collected for determination of catecholamines (Anton and Sayre 1963), ^{3}H- and ^{35}S activities (liquid scintillation counting) and of hexosamine (Cessi and Piliego 1960)

Results

Endogenous or exogenous (^{3}H-activity) catecholamines and ^{35}S-activity showed very similar distribution patterns, the vesicle-containing fraction exhibiting a marked

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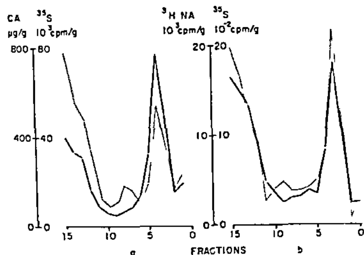


Fig 1 Centrifugation of homogenates from dog adrenal medulla in continuous sucrose density gradient (0 25—2 2 M), ascorbic acid 10 µg/ml EDTA 1 mg/ml 120 000 × g 30 min 2° C
a) Distribution of endogenous catecholamines ——— and of ^{35}S-activity
b) Distribution of ^{3}H NA ——— and of ^{35}S activity

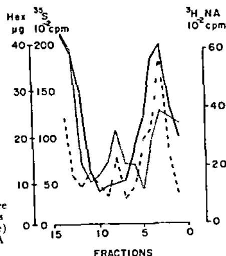


Fig 2 Distribution in continuous gradient (see Fig 1) from homogenized dog medulla of hexosamine (Hex) (measured as galactosamine) of ^{35}S and of ^{3}H NA activity ———

^{35}S-activity peak (Fig 1 a and b) Essentially identical results were obtained from cat (6) and dog (9) adrenals Our assumption that the ^{35}S activity reflected the presence of an SMPS is supported by the uptake in the gradient vesicle fraction of Alcian blue—a dye used for specific staining of sulphomucopolysaccharides—and by the similar distribution of hexosamine and ^{35}S (Fig 2) Catecholamine containing vesicles from homogenized bovine adrenal medullas were found to contain an SMPS-protein complex, which on column chromatography (Dowex 1 X2) was split

into two components, a basic protein and an SMPS, tentatively identified as chondroitin sulphate. The content of the SMPS was calculated to be about 20—25 mg/g adrenal medulla dry weight (to be published). The SMPS protein complex is able to bind adrenaline and sodium. Quantitative studies on the binding capacity are in progress.

Comments

As mentioned above, Hi and 5 HT-containing granules from rat mast cells and rat thrombocytes have been found to contain an SMPS protein complex capable of reversibly binding cations and biogenic amines. The present study indicates the occurrence of an SMPS protein complex in the catecholamine containing vesicles of the adrenal medulla from cats, dogs and cows.

Studies in progress in our laboratory have shown that also adrenergic and cholinergic vesicles contain an SMPS protein complex. Our data on the chemical properties and the amine binding abilities of these SMPS protein complexes are still only fragmentary. If it can be confirmed that they show amine storing properties similar to those of the corresponding complexes in mast cell and thrombocyte granules they may play an important role in the storage and release of biogenic amines in adrenergic and cholinergic mechanisms. Further studies designed to show whether or not SMPS protein complexes have such a generalized storage function regarding biogenic amines and possibly other positively charged biogenic substances are in progress.

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Acta physiol. scand. 1971. 83. 289—299

From the Division of Physiological Chemistry, Chemical Center, University of Lund, Sweden

Uptake of Micellar Lipids by Small Intestinal Segments [under Different Experimental Conditions].

By

CHRISTER SYLVÉN

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Abstract

SYLVÉN, C *Uptake of micellar lipids by small intestinal segments (under different experimental conditions)* Acta physiol scand 1971 *83* 289—299.

Uptake of radioactive cholesterol, sitosterol and monoolein from micellar solutions was studied under different conditions in rat small intestinal segments both with and without blood supply, and in inverted [illegible] lution did not in- [illegible] s was maximal at [illegible] 1) A high uptake [illegible] a low uptake of sitosterol with a high uptake of cholesterol The latter state corresponds to normal uptake *in vivo* These states were demonstrated under different experimental conditions involving exclu[illegible] [illegible] of uptake

In vivo the intestinal absorption of sitosterol in the rat amounts to 3—6 per cent of the dose administered This fraction is absorbed largely independent of the dose fed The absorption of cholesterol has the same characteristics but a larger fraction of about 40 per cent is absorbed Both sterols are absorbed independently and do not seem to influence each other's absorption competitively (Sylven and Borgstrom 1969) The difference in the absorption has indirectly been traced to different rates of flow of sterol from the micellar phase through the plasma membrane of the epithelial cell into the cell (Sylven and Nordstrom 1970)

In vitro, however, both sterols are taken up to the same degree by everted sacs (Feldman and Borgstrom 1966) Thus the specificity in sterol uptake found *in vivo* does not seem to be manifested in *in vitro* preparations

Sterol absorption was therefore studied in small intestinal segments *in situ* with and without blood supply (Sylven 1970) These preparations thus represented a gradual transition from *in vivo* to *in vitro* conditions Uptake of sterols and its specificity seemed to be an energy-dependent process dependent on blood supply

In this investigation an attempt has been made to characterize this process further. Its dependence on a Na^{+}/K^{+}-pump and temperature has been investigated. As absence of blood supply changed the specificity of uptake the question of reversibility was studied. Bile salts are known to be required for cholesterol absorption (Treadwell and Vahouny 1968). Therefore their influence on uptake has also been investigated.

Methods

The materials used were identical to those used before (Sylvén 1970). In addition sodium taurochenodeoxycholic acid (NaCDC) was used. It was obtained from Maybridge Chemical, Tintagel, Cornwall, U.K.

Uptake of different micellar lipids by intestinal segments with and without blood supply was studied as described before (Sylvén 1970). However, in this investigation the direct uptake experiments were preceded by different kinds of treatment of the animal.

1. *Temporal exclusion of blood supply to the small intestine*

The rat fasted from the day before was anaesthesized with Nembutal. The abdominal wall was opened. The small intestine was tied off both at the level of the duodeno-jejunal and at the level of the ileo-caecal junction. The superior mesenteric artery was temporarily interrupted with bulldog clamps at the same levels. After different time intervals the blood supply was released and uptake studied as before.

2. *Hypothermic treatment*

The fasted and anaesthesized rat was put into an ice bath. The rectal temperature was observed. One degree above the desired temperature the animal was taken up from the ice bath. When warming the rat was desired it was done by aid of an infra red lamp. At temperatures below 25° C the rat was artificially ventilated 10—12 times per minute. 95 % O_2 5 % CO_2 directly from a gas tube via a rubber tube was gently pressed through the nose of the animal so that the chest gently expanded.

When having reached the desired temperature the abdominal wall was opened. A thermometer was placed at the bottom of the abdominal cavity. Isotonic saline of the desired temperature was constantly poured over the small intestine from a Pasteur pipette. Uptake experiments were performed as before with micellar solutions of the desired temperature.

3. *Biliary drainage*

Fasted rats were provided with an external bile fistula the day before experimentation. Under ether anaesthesia the abdominal wall was opened and a thin polyethylene tube was inserted into the bile duct proximal to the pancreas.

After operation the animal was put into a Bollman cage with free access to water containing 0.61 % NaCl and 0.04 % KCl. The next day, 24 hrs after operation, experiments were done as before.

In one experiment part of the small intestine was excised from the anaesthesized animal and carefully transferred into a 37° C bath with 95 % O_2 5 % CO_2 saturated Krebs-Ringer phosphate buffer containing 10 μmoles glucose/ml. Inverted sacs were prepared and 0.4 ml micellar solution injected into each. Thereafter the sac was immediately transferred to a bath containing the same oxygenated buffer solution but of different temperatures. 15 min after injection the segment was isolated and processed as before.

The micellar solutions were prepared according to Hofmann (1963). They contained different concentrations of NaTDC, NaCDC or NaTC in phosphate buffer pH 6.3 that was 0.15 M with respect to Na. The solutions were either 2 mM or 10 mM with respect to monoolein and contained also radioactive tracers of cholesterol-4-^{14}C, cholesterol-^{3}H, sitosterol-22,23-^{3}H or monoolein-^{14}C. Tracers and monoolein were taken to dryness and the buffered bile salt solution added. Shaking was performed until a water-clear solution was obtained.

Results

Uptake and its dependence on the Na/K ratio of the micellar test solution

Fig 1 shows that the amount of radioactive monoolein and cholesterol taken up did not change when the Na/K ratio of the micellar test solution was changed Similar results were obtained when the administered radioactive pair was sitosterol ^{3}H and cholesterol ^{14}C Irrespective of the Na/K ratio used the ratio between the uptakes of sitosterol and cholesterol was 0 30—0 40 when the sterol ratio in the micellar test solution was taken as 1 00 This ratio of uptake corresponds to that found in the normal animal (Sylven 1970 Borgstrom 1968)

Uptake and its dependence on blood supply

The blood supply to the whole small intestine was excluded for different times after which it again was restored After different times of recovery uptake was studied in an intestinal segment for 15 min Fig 2 shows that the ratio increased with increasing time of exclusion of blood supply The ratio was constant and independent of time of recovery This indicates that irreversible damage to the uptake process had been caused by the absence of blood supply Fig 3 represents an experiment where the blood supply to the small intestine was excluded for 30 min After this time the clamp was released and sterol uptake was studied in an intestinal segment with and without blood supply In both kinds of preparations the ratio of uptake was 1 0 However uptake into a segment with blood supply was lower than that found in a segment without blood supply

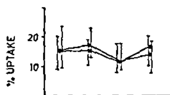
Uptake and temperature

The uptake was studied in preparations that were isolated rapidly and with care and transferred into oxygenated solutions at different temperatures The ratio of uptake into inverted sacs is shown in Fig 4 It was about 0 70 for all temperatures tested The amount of radioactivity taken up in all cases did not exceed three per cent

Fig 5 illustrates uptake studied in hypothermic animals *in vivo* Maximal uptake of both sterols was found at 37° C At 41° C a decrease for both sterols was observed Lowering the temperature from 37° C to 25° C resulted in a lowered uptake for both sterols with a Q_{10} of about 3 suggesting a metabolic process The uptake did not reach zero At lower temperatures the amount of cholesterol radioactivity taken up was of the same order as for 25° C At temperatures of 25° C and above the ratio of uptake was about 0 4 Below 25° C the ratio increased towards 1 0 thus giving an uptake of sitosterol of almost the same magnitude as that of cholesterol

With excluded blood supply in hypothermic animals the ratio increased for all temperatures tested to 0 70—0 90 i e apparently that found in *in vitro* preparations (Fig 4) In contrast to the low and non temperature dependent uptake *in vitro* in this case there was a marked uptake that increased with temperature

These results raised the question whether the rise in the ratio obtained at low temperatures was irreversible Animals were made hypothermic and thereafter re[illegible]



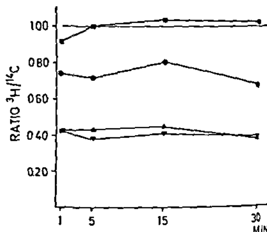


Fig 1

Fig 2

Fig 1 Uptake of a cholesterol ^3H tracer ± range ▼—▼ and monoolein ^{14}C ± range ●—● by an intestinal segment with blood supply when the Na^+/K^+ ratio of the micellar solution was changed 15 min before isolation of the segment 0 4 ml of the micellar solution was injected into the segment The solution containing the cholesterol ^3H tracer was 10 mM NaTDC and 10 mM monoolein in phosphate buffer pH 6 3 The total concentration of Na^+ and K^+ was 0 15 M In each of the four rats used four determinations with changing Na^+/K^+ ratio were made

Fig 2 Ratio between the uptake of a sitosterol ^3H and of a cholesterol ^{14}C tracer in an intestinal segment when the ratio of the micellar solution was 1 00 The blood supply to the whole small intestine was excluded for 1 min ▼—▼ 5 min ▲—▲ 15 min ●—● and 30 min ■—■ Thereafter the blood supply was restored At different times after release 0 4 ml of the micellar solution was injected into an intestinal segment that was isolated 15 min later The micellar solution containing the tracers was 10 mM NaTDC 10 mM monoolein and 0 15 M Na^+ in phosphate buffer pH 6 3 For each period of exclusion of blood supply four rats were used and in each rat four determinations were made

to 37° C Two temperatures were used 15 5° C (Fig 6) and 18 5° C They gave identical results After rewarming the high ratio was reversed to a low ratio (0 30—0 40) in segments with blood supply Segments with excluded blood supply also gave a ratio earlier found in animals that were not pretreated The change in the ratio of uptake reflected an increased uptake of cholesterol while uptake of sitosterol at 37° C was of the same size or lower as that found at the lower temperature The uptake of cholesterol in the rewarmed animal was close to 20 per cent while in non pretreated animals it was about 40 per cent

Uptake and bile salts

Uptake was studied in rats with external bile fistulae prepared 24 h before the experiment Micellar test solutions containing radioactive lipids and increasing concentrations of NaTC NaTDC or NaCDC were injected into small intestinal segments These were isolated 10 min later NaTDC and NaCDC had identical effects on uptake Therefore experiments with NaCDC are not given Fig 7 shows that the ratio of sterol uptake was 0 70—0 90 for a low concentration of both NaTDC and

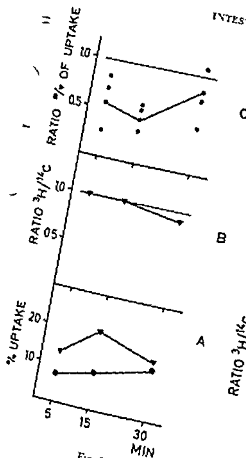


Fig 3

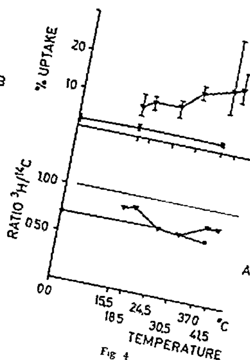


Fig 4

Fig 3 *A* Uptake of a cholesterol-^{14}C tracer with time in an intestinal segment without ▼—▼ and with ●—● blood supply. Before experimentation the blood supply to the whole small intestine was excluded for 30 min. Thereafter the blood supply was released and the experiment performed. Into each segment 0.4 ml of a micellar solution was injected. This contained tracers of sitosterol-^{3}H and cholesterol-^{14}C and was 10 mM NaTDC, 10 mM monoolein and 0.15 M Na in phosphate buffer pH 6.3. In each of the four rats used six determinations were made.

B Ratio between the uptake of the sitosterol-^{3}H and of the cholesterol-^{14}C tracer in the experiment related in A. The ratio was in the micellar solution 1.00.

C Ratio between uptake of cholesterol in segments with blood supply and in segments without blood supply in the experiment related in A.

Fig 4 *A* Ratio of uptake between a sitosterol-^{3}H and a cholesterol-^{14}C tracer during different temperatures when the ratio was 1.00 in the micellar solution. ●—● refers to *in vitro* uptake by an inverted intestinal sac prepared *in vitro* and ▼—▼ to uptake by a segment without blood supply in a rat made hypothermic to the indicated temperature ± 1.0° C. 0.4 ml of a micellar solution was injected into the intestinal preparation that was isolated 15 min later. The micellar solution containing tracers of sitosterol-^{3}H and cholesterol-^{14}C was 10 mM NaTDC, 10 mM monoolein and 0.15 M Na in phosphate buffer pH 6.3. *In vitro* the intestinal sac was bathing in a 95 % O_2, 5 % CO_2 saturated Krebs-Ringer phosphate buffer of the indicated temperature. The solution contained 10 μmoles glucose/ml. Each point represents four determinations made in preparations in or from different rats.

B Per cent uptake ± range of the cholesterol-^{14}C tracer in the experiment related in A.

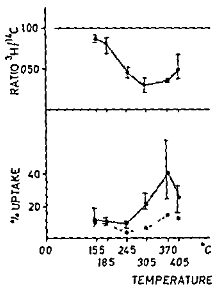


Fig 5

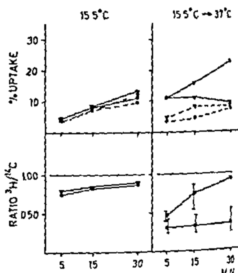


Fig 6

Fig 5 *A* Per cent uptake ± range of a cholesterol-^{14}C tracer ●—● and per cent uptake of a sitosterol-^{3}H tracer ●---○ in an intestinal segment with blood supply in a rat made hypothermic to the indicated temperature ± 1 0° C 30 min before isolation of the segment 0 4 ml of a micellar solution was injected The solution containing the tracers was 10 mM NaTDC, 2 mM monoolein and 0 15 M Na⁺ in phosphate buffer pH 6 3 Each point represents four determinations made in different rats

B Ratio between the uptake of the sitosterol ^{3}H and of the cholesterol-^{14}C tracer in the experiment related in A The ratio of the micellar solution was 1 00

▼—▼ cholesterol-^{14}C } in segments without blood supply
▼-- ▼ sitosterol-^{3}H }

●—● cholesterol ^{14}C } in segments with blood supply
●---● sitosterol ^{3}H }

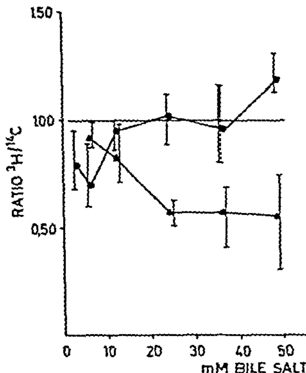
0 4 ml of a micellar solution was injected into the segment The solution containing the sterol tracers was 10 mM NaTDC 2 mM monoolein and 0 15 M Na⁺ in phosphate buffer pH 6.3 In each experiment six determinations were made in each of the four rats used

Below The ratio between the uptake of the sitosterol ^{3}H and the cholesterol ^{14}C tracer in the experiment related above The ratio in the micellar solution was 1 00

NaTC. Increasing concentration of NaTDC produced an increase in the ratio to about 1 00 while NaTC gave a decrease to values between 0 50 and 0 60

When NaTDC was used the uptake of radioactive cholesterol was highest (25 per cent) for the lowest concentration used With increasing concentration the uptake decreased first rapidly and then slower reaching 10 per cent for 18 mM NaTDC As the ratio of uptake was largely 1 00 the uptake of cholesterol also represents that of sitosterol The uptake of monoolein was slightly higher than that of cholesterol but followed the same trend This is shown in Fig 8 that also shows the uptake

Fig 7 Ratio ± range of uptake of a sitosterol ^{3}H and a cholesterol ^{14}C tracer with changing micellar bile salt concentration. The uptake was studied in an intestinal segment with blood supply. The bile salts used were ●——● NaTDC and ▲——▲ NaTC. 10 min before isolation of the segment 0.4 ml of a micellar solution containing the tracers and the bile salt was injected. In addition the solution was 2 mM monoolein and 0.15 M Na^{+} in phosphate buffer pH 6.3. Five or six determinations were made in each of the four rats used. The rats were provided with external bile fistulae



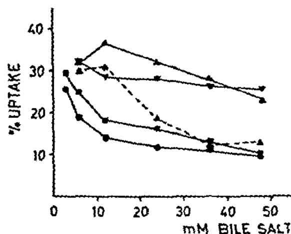


Fig 8 Per cent uptake of the radioactive sitosterol and of the radioactive cholesterol tracer in the experiment related in the legend to Fig 7. In addition the figure shows the uptake of radioactive monoolein under in other respects identical experimental conditions

●—● cholesterol, ■—■ monoolein } from micellar solution containing NaTDC

▲—▲ cholesterol, ▲ ▲ sitosterol, ▼—▼ monoolein } from micellar solution containing NaTC

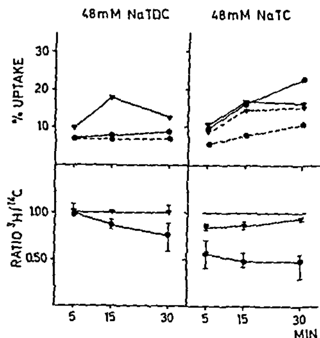


Fig 9 *Above* Uptake of a cholesterol ^{14}C and a sitosterol ^{3}H tracer with time in an intestinal segment with and without blood supply in rats provided with external bile fistulae 0.4 ml of a micellar solution was injected into the segment The solution containing the sterol tracers was 48 mM NaTDC (to the left) or NaTC (to the right) 2 mM monoolein and 0.15 M Na$^+$ in phosphate buffer pH 6.3

▼—▼ cholesterol ^{14}C, ▼ ▼ sitosterol ^{3}H } in segments without blood supply

●—● cholesterol ^{14}C, ● ● sitosterol ^{3}H } in segments with blood supply

In each experiment six determinations were made in each of the four rats used
Below The ratio between the uptake of the sitosterol ^{3}H and the cholesterol ^{14}C tracer in the experiment related above The ratio was in the micellar solution 1.00

when NaTC was used At the lowest concentration it was of the same size (30 per cent) as that obtained when NaTDC was used Increasing concentration of bile salt gave an uptake of cholesterol that first increased and thereafter decreased moderately The uptake of monoolein was of the same size as that found for cholesterol At a low concentration of NaTC uptake of sitosterol was in the same range as for cholesterol With increasing concentration however it decreased markedly down to the level of sterol uptake obtained when a high concentration of NaTDC was used

The effect of NaTDC versus NaTC was further studied in uptake experiments during different times This is shown in Fig 9 When 48 mM NaTDC was used the ratio of uptake was 1.00 in segments without blood supply The corresponding uptake was close to 15 per cent after 15 or 30 min With intact blood supply the ratio was still high but decreased with time reaching a value of about 0.80 after 30 min The presence of blood supply resulted in a decreased amount of radioactivity taken up to between 5 and 10 per cent After 5 min there was no net uptake with time When 48 mM NaTC was used in segments without blood supply a ratio between 0.80 and 1.00 was obtained The amount of radioactivity taken up was equal to the

TABLE I Different states of sterol uptake obtained during different experimental conditions 1 and 5 refer to previously published experiments (4)

Preparation	Ratio 0 80—1 00 High uptake	Ratio 0 70—1 00 Low uptake (appr 5—15 %)	Ratio 0 30—0 50 High uptake (appr 15—40 %)
1	*In vitro* preparation		
2	Exclusion of blood supply for 30 min Uptake under prolonged exclusion	Anoxic pretreatment for 30 min Uptake with released blood supply	
3	Bile drainage Uptake under excluded blood supply High concentration of NaTDC	Bile drainage Uptake with intact blood supply High concentration of NaTDC	
4	Bile drainage Uptake under excluded blood supply High concentration of NaTC		Bile drainage Uptake with intact blood supply High concentration of NaTC
5		*Preparation without pre* treatment Uptake under excluded blood supply	*Preparation without pre* treatment Uptake with intact blood supply
6		Hypothermic pretreatment	Hypothermic pretreatment and rewarming

found when NaTDC was used in the same kind of preparation The prescence of blood supply resulted in a decreased ratio close to 0 50 As in the experiment where the effect of increasing concentration of bile salt was studied this change in ratio was due to the fact that the sitosterol uptake decreased to the level of sterol uptake seen when NaTDC was used However, when NaTC was used there was an increased uptake with time The uptake of cholesterol radioactivity also increasing with time was of the same size as that found in segments with excluded blood supply irrespective of whether NaTDC or NaTC was used

In Table I an attempt has been made to summarize the present findings In general three states of sterol uptake were found The first state characterized by a high ratio of uptake and generally a high uptake was found in four cases In *in vitro* preparations it was found with or without oxygenation while in the following three it was found only in segments with excluded blood supply The prescence of blood supply resulted for preparations 2 and 3 in the finding that the state of uptake was changed to the second one characterized by a low uptake but a high ratio of specificity Preparation 4 was changed to the third state of uptake i e that ordinarily found *in vivo* It is characterized by a high uptake of cholesterol and a low ratio Without blood supply and under hypothermia respectively preparations 5 and 6 were in the second state of uptake The prescence of blood supply and rewarming respectively resulted in a normal state of uptake

Discussion

In the previous investigation (Sylvén 1970) it has been shown that uptake of sterols by small intestinal segments with blood supply increased with time. The ratio of uptake was of the same size (0.40) as that found *in vivo*. Exclusion of blood supply resulted in that the ratio increased approximately to 0.70 with no net uptake of sterols five min after administration of the micellar test solution. When intestinal slices were incubated *in vitro* there was a marked net uptake with time while the ratio in this case was about 1.00. By administration of KCN in the micellar test solution the role of blood supply could be further related to an energy dependence. These results suggested that intestinal absorption of sterols is an active process. However, the data do not suggest an active carrier mechanism. Such a mechanism could not explain the fact that there was no net uptake into intestinal segments without blood supply while a marked uptake was seen in *in vitro* preparations. In order to explain the results obtained it was necessary to look at the membrane as a dynamical four dimensional space time system.

In the present study it has been demonstrated that during different experimental conditions an uptake is obtained that has the same characteristics as that previously found *in vitro*. The *in vitro* uptake was shown to have the characteristics of a diffusion process (Feldman and Borgström 1966), probably dependent on the physico-chemical properties of the intestinal preparation. This state of uptake may be changed via a second state to the third state found in the normal animal. The characteristics of these three states of uptake involve in sequence a high uptake of both sterols, a low uptake of both sterols and a preserved low uptake of sitosterol while the uptake of cholesterol increases. The uptake can be both irreversibly and reversibly influenced. It was dependent on parameters affecting the interior of the cell (blood supply and hypothermia) but also the exterior of the cell (increasing concentrations of NaTDC or NaTC). These experimental facts together with the further fact that the uptake was not dependent on a Na/K pump (the uptake was not influenced by different Na/K ratios in the micellar test solution) lead to the possible interpretation that the active character of the uptake process is not directly correlated with the particular transfer of each molecule. The function seems to be a coordinated complex activity of the plasma membrane. When some single activity of this complex is changed the coordinated activity changes its state reversibly. At more fundamental changes the coordination is irreversibly damaged so that the activity cannot reach its highest state. The principle responsible for the specificity in sterol absorption therefore cannot be ascribed to a particle in the form of a molecule. To find an *entity* that *contains* the whole phenomena one is forced to look at the process in a fourdimensional space time system as previously discussed (Sylvén 1970).

It may be that organisational forces are operative here; such forces are not directly accessible to experimental verification at the present time. Alternatively, therefore, the character of the specificity mechanism in sterol absorption may be described as an expression of the living active cell with all its parts dynamically interrelated in

order to normally carry out the specific sterol uptake and lipid absorption The dynamic arrangement of lipid molecules in the cell may be sensitive to disturbances in this living organisation If the phenomenon is described in these terms — and the hypothesis can be confirmed — intestinal lipid uptake should be viewed to be a truly physiological phenomenon

It was previously shown (Sylven 1970) that uptake of sterols by the living cell had a close relation to the uptake of other species of lipid Uptake of every species tested was dependent on blood supply in a similar manner to the uptake of sterols In this investigation the uptake of monoolein had the same characteristics as the uptake of cholesterol It was not influenced by the Na/K' ratio of the micellar test solution and it was dependent on bile salts in the same way as cholesterol was The results may indicate that lipid uptake generally has the same characteristics as uptake of sterols

The normal transfer of lipids through the plasma membrane then partially should be dependent on lipid solubility and the physico-chemical properties of the membrane and partially on fundamentally physiological properties as discussed above The importance of the physico-chemical aspect is demonstrated by the fact that uptake in state one and three is of the same magnitude

It was found that a high concentration of NaTC was necessary for normal absorption This correlates well with findings indicating that a large quantity (10 mg/100 g rat) of bile acids is released per unit time in the rat (Shefer, Hauser, Bekersky and Mosbach 1969)

This project was supported by grants from the Swedish Nutrition Foundation and from the Medical Faculty University of Lund (to C Sylven) and by grants from the Scientific Council of the Swedish Margarine Industry (to prof B Borgstrom)
Mrs H Lundberg provided skilful technical assistance The author wish to thank Dr F A Green for helpful suggestions in preparing the manuscript

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Discussion

In the previous investigation (Sylven 1970) it has been shown that uptake of sterols by small intestinal segments with blood supply increased with time The ratio of uptake was of the same size (0 40) as that found *in vivo* Exclusion of blood supply resulted in that the ratio increased approximately to 0 70 with no net uptake of sterols five min after administration of the micellar test solution When intestinal slices were incubated *in vitro* there was a marked net uptake with time while the ratio in this case was about 1 00 By administration of KCN in the micellar test solution the role of blood supply could be further related to an energy dependence These results suggested that intestinal absorption of sterols is an active process However, the data do not suggest an active carrier mechanism Such a mechanism could not explain the fact that there was no net uptake into intestinal segments without blood supply while a marked uptake was seen in *in vitro* preparations In order to explain the results obtained it was necessary to look at the membrane as a dynamical four dimensional space time system

In the present study it has been demonstrated that during different experimental conditions an uptake is obtained that has the same characteristics as that previously found *in vitro* The *in vitro* uptake was shown to have the characteristics of a diffusion process (Feldman and Borgstrom 1966) probably dependent on the physico-chemical properties of the intestinal preparation This state of uptake may be changed via a second state to the third state found in the normal animal The characteristics of these three states of uptake involve in sequence a high uptake of both sterols a low uptake of both sterols and a preserved low uptake of sitosterol while the uptake of cholesterol increases The uptake can be both irreversibly and reversibly influenced It was dependent on parameters affecting the interior of the cell (blood supply and hypothermia) but also the exterior of the cell (increasing concentrations of NaTDC or NaTC) These experimental facts together with the further fact that the uptake was not dependent on a Na/K pump (the uptake was not influenced by different Na/K ratios in the micellar test solution) lead to the possible interpretation that the active character of the uptake process is not directly correlated with the particular transfer of each molecule The function seems to be a coordinated complex activity of the plasma membrane When some single activity of this complex is changed the coordinated activity changes its state reversibly At more fundamental changes the coordination is irreversibly damaged so that the activity cannot reach its highest state The principle responsible for the specificity in sterol absorption therefore cannot be ascribed to a particle in the form of a molecule To find an *entity* that *contains* the whole phenomena one is forced to look at the process in a fourdimensional space time system as previously discussed (Sylven 1970)

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Methods

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factor in the linear regression analysis Calculations were performed on a PDP 8 (Digital Equipment Corp USA) with the aid of a FOCAL-program (Eriksson 1971) Drugs used were Atropine sulphate, Methylatropine nitrate and Morphine hydrochloride purchased from the Military Pharmacy (Sweden), α Dinitrophenol and Hexamethonium chloride (Merck, Germany), N ethylmaleimide (Schucher, Germany), Decamethonium bromide (Fluka Switzerland), choline chloride (Kebo, Sweden), and Promethazine chloride (Recip, Sweden)

Results

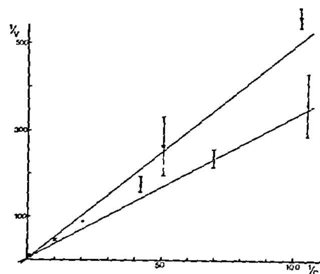
General

Mean weight of choroid plexus from the lateral ventricles was 7 0 mg ± SD 0 2 mg (n = 122) and fourth ventricle plexus weight was 5 8 mg ± SD 0 6 (n = 36) Choroid plexus swelling during incubation as estimated from weight before and after incubation was below 10 % Routine histological examination[3] revealed no important differences in tissue structure before and after incubation Results are given as the tissue/medium concentration ratio (T/M = dpm per gram tissue wet weight/dpm per ml incubation medium) calculated without correction for extracellular space or incubation swelling Fourth ventricle plexa are treated separately High voltage electrophoresis revealed no metabolites in choroid plexus extract or in incubation medium after 60 min of incubation with either drug, when investigated by paper scanning (Model 7200 Packard Instruments, USA)

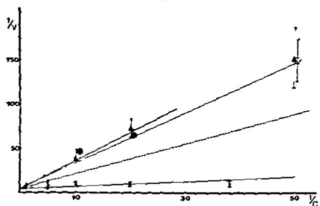
[1] T atropine generally labelled 434 mC/mmol), was purchased from the Radiochemical Centre Amersham England T methylatropine iodide was synthesized from T atropine as described elsewhere (Albanus Sundwall and Vangbo 1969)

[2] 14 ml of a solution consisting of dioxane 960 ml PPO 5 12 g POPOP 128 mg and Naphtalene 102 4 g or 12 ml of Toluene Triton X 100 (500 ml of each respectively with Omnifluor® 4 g, NEN Corp Mass USA

[3] Formaldehyde fixation paraffin embedding and haematoxylin eosin or Van Gieson s



a



b

Fig 3 Double reciprocal plot (Lineweaver Burk) of uptake velocity V in mmole × kg⁻¹ × min⁻¹ versus incubation medium concentration C in mM Choroid plexus from lateral ventricles Vertical bars indicate SD of 1/V

a) Upper line atropine with morphine 10^{-3} M ✦ Lower line atropine alone ○

b) Lines in order from top methylatropine with choline 5 mM ● atropine 1 mM ▲ hexamethonium 1 mM ○ and methyl atropine alone

atropine Omission of glucose or oxygen in the incubation medium and incubation at 0—4° C also reduced uptake considerably The results are summarized in Table I

Atropine and methylatropine uptake versus other drugs

Atropine uptake was decreased in the presence of other tertiary amines as morphine and promethazine but also by the quaternary amines hexamethonium and methyl atropine (Table I A Lineweaver Burk plot of the influence of morphine on atropine uptake and calculated V_{max} suggest competitive inhibition with an inhibitor coefficient of 6.2×10^{-4} M Fig 3 Table II

Methylatropine uptake is decreased by the other quaternary amines hexamethonium decamethonium and choline Table I) Hexamethonium and choline interfere with methylatropine uptake by competitive inhibition (Fig 3 Table II

TABLE I Inhibition of Atropine and Methylatropine uptake

Drug mM	Inhibitor	% Inhibition of uptake ± SD (n)[1]
Atropine		
0 1	N-ethylmaleimide 0 1 mM	77, 84
0 1	Glucose omitted	43, 16
0 1	Incubation at 0—4° C	83±3 (4)
0 1	Incubation without O_2	86, 88
0 0004	Choline 0 001 mM	21±9 (3)
0 1	Hexamethonium 1 0 mM	40, 25
0 01	Methylatropine 0 1 mM	39±13 (5)
0 02	Promethazine 0 2 mM	78, 66
Methyl atropine		
0 1	N ethylmaleimide 0 1 mM	97±1 (3)
0 1	Dinitrophenol 0 1 mM	58, 65
0 1	Glucose omitted	78, 91
0 1	Incubation at 0—4° C	98±1 (4)
0 1	Incubation without O_2	92 5
0 1	Decamethonium 1 0 mM	82, 78
0 1	Morphine 1 0 mM	61, 68

TABLE II Competitive inhibition of atropine and methylatropine uptake

	Inhibitor mM	V_{max}±SE mmol min×kg	K_m K_p±SE mM	K_i[x] mM	Number of exp
Atropine	—	0 35±0 002	0 93±0 12	—	40
	Morphine 1 0	0 59±0 17	2 43±0 97	0 62	6
Methylatropine	—	0 26±0 02	0 075±0 02	—	30
	Atropine 1 0	0 17±0 02	0 48±0 14	0 19	10
	Choline 5 0	0 25±0 07	0 66±0 35	0 64	6
	Hexamethonium 1 0	0 37±0 12	0 39±0 30	0 24	4

x calculated as $\dfrac{\text{inhibitor concentration}}{\dfrac{K_p}{K_m} - 1}$

As expected, uptake is inhibited by atropine and morphine Table I Also the inhibition by atropine appears to be competitive (Fig 3, Table II

Dog choroid plexa

In 2 expts adult beagle, instead of rabbit choroid plexa were used Methods were the same as for the rabbit tissue except that the dogs were killed with an overdose

[1] When two values or less individual c

of pentobarbital. At a medium concentration of 10^{-5} M, T/M was 6±SD 3 for atropine and 6±SD 1.5 for methylatropine after 40 min of incubation with choroid plexa from the lateral ventricles.

Discussion

Rabbit choroid plexa accumulate atropine and methylatropine by a mechanism which satisfies the conventional criteria for active transport, i.e. accumulation against a concentration gradient by an energy dependent saturable process. Besides active transport, there is a passive diffusion of drug into the tissue from the incubation medium. An estimate of the size of this part of the uptake can be obtained at very high concentrations of the drug in the incubation medium when active transport though running at maximal speed contributes relatively less to tissue concentration. This is only correct as long as the high concentrations of the drugs do not change the properties of the tissue. As seen from Fig. 2 the T/M is 1.2 at 10^{-2} M incubation medium concentration. If this ratio is substracted from all T/M values when calculating, a too low estimate of V_{max} is obtained, 230 and 250 μmol × kg^{-1} × min^{-1} for atropine and methylatropine, respectively. At incubation medium concentration of 10^{-2} M diffusion would thus maximally account for $1.2 \times 10^{-2} - 3.0 \times 230 \times 10^{-6} = 0.5 \times 10^{-2}$ M and $1.2 \times 10^{-2} - 4.0 \times 250 \times 10^{-6} = 0.2 \times 10^{-2}$ M of choroid plexus concentration for atropine and methylatropine respectively. Taking into account the hight T/M's obtained already at 10^{-3} M, a correction for diffusion is not considered necessary when excluding the values from 10^{-2} M from the kinetic calculations.

The kinetic data for the uptake is compatible with a carrier mechanism which is the same for both drugs. Further, this uptake mechanism is shared with some other tertiary and quaternary drugs and the endogenous substance choline. The transport system for quaternary amines has been shown to be responsible also for the transport of some primary amines including some transmittor substances (Tochino and Schanker 1965 b). Because of the considerable variation in uptake velocity at the different incubation concentrations used, the standard deviations of K_m and V_{max} are large and the interpretation of the data must be made with caution. It is thus not possible to exclude with certainty that more than one transport system is involved. Different transport systems for tertiary and quaternary amines were suggested by Takemori and Stenwick (1966) who obtained no inhibition of choroid plexus uptake of morphine by hexamethonium. Such an inhibition would be expected from our results since morphine competitively inhibits atropine transport and both atropine and hexamethonium are competitive inhibitors of methylatropine transport. Further morphine decreases methylatropine accumulation.

The rabbit choroid plexus transport system for quaternary ammonium compounds reported in this paper has also been found in the dog, cat and guinea pig (Tochino and Schanker 1965 a).

It seems reasonable to assume that the transport mechanism *in vitro* operates in the ependymal vascular direction since the vascular surface exposed to incubation

medium must be extremely small It has also been shown that organic acid dyes such as phenol red and chlorophenol red, are taken up by the choroid plexus *in vitro* and the dye is accumulated in the capillaries (Rall and Sheldon 1961) Further, in the perfused horse choroid plexus glucose is transported from the incubation medium into the perfusate against a concentration gradient (Csáky and Rigor 1968)

It has been shown that certain substances besides the earlier mentioned quaternary amines (Shanker *et al* 1962) are cleared out of the cerebrospinal fluid (CSF) partly by a carrier mediated mechanism, *i e* iodypyracet and phenolsulphonphthalein (Pappenheimer, Heisey and Jordan 1961), iodide (Becker 1961, Pollay and Davson 1963), thiocyanate and PAH (Pollay and Davson 1963) sulphate (Lorenzo, Cutler and Barlow 1967, Robinson *et al* 1968 a) penicillin (Dixon Owens and Rall 1969), some neutral amino acids (Snodgrass *et al* 1969), lysine (Cutler 1970) and glucose (Bronsted 1970) As discussed above, this is possibly also the case with atropine and methylatropine Choroid plexa *in vitro* accumulate some of these substances by a mechanism which fulfils the requirements for active transport (Becker 1961, Welch 1962 a, Tochino and Schanker 1965, Welch 1962 b, Robinson *et al* 1968 b, Kaplan and Pollay 1968, Lorenzo and Cutler 1969, Csáky and Rigor 1964) It thus seems possible that a carrier mediated CSF clearance mechanism is partly localized to the choroid plexus

Davson (1963) suggested that when CSF has a lower concentration of a substance than brain extracellular water, it acts as a sink for the clearance of that substance from the brain Such brain — CSF gradients have been shown to exist for iodide thiocyanate, the weak acid 5 5 dimethyl oxazoladine 2 4 dione (DMO) and sucrose When CSF clearance of these substances is reduced brain concentration rises (Bito Bradbury and Davson 1966 Coben 1969, Pollay and Kaplan 1970 Rollins and Reed 1970, Oldendorf and Davson 1967) Further, in the case of thiosulphate, concentration in the brain hemisphers falls towards the CSF spaces after i v infusion of the substance (Pollay and Kaplan 1970) Such gradients have been observed 2 and 10 h after s c injection of atropine (Albanus and Winbladh unpublished observations) Whether a CSF sink has any importance for the clearance of atropine from the brain is however not elucidated

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Uptake of Serotonin in Blood Platelets in vitro. III: Effects of Acetate and Other Monocarboxylic Acids

By

O. Lingjærde, Jr

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Abstract

Lingjærde, O., Jr. *Uptake of serotonin in blood platelets in vitro III Effects of acetate and other monocarboxylic acids* Acta physiol scand 1971 *83* 309—318

The chloride-dependent uptake of serotonin in human blood platelets is stimulated by acetate,
n the absence of
The stimulatory
onic site' of the
'activating site"
on the plasma membrane in the same way as was previously shown to be the case for nitrite The specific activating effect of both acetate and nitrite is blocked by increasing the extracellular potassium concentration to about 30 mM At pH 6.2, acetate in concentrations higher than about 10 mM exerts an uncompetitive inhibitory effect on serotonin uptake This effect is not counteracted by potassium, and seems to be quite independent of the stimulatory effect. The stimulatory effect of acetate on serotonin uptake is shared by other monocarboxylic acids, such as formate, propionate, butyrate, lactate and pyruvate The possibility that lactate may act as a physiological regulator of serotonin uptake in platelets and "serotoninergic" neurons is mentioned

The uptake of serotonin (5 hydroxytryptamine, 5HT) in human blood platelets *in vitro* is dependent on the presence of chloride, or some other small anion in the incubation medium (Lingjærde 1969, 1971 a, 1971 b) Larger anions such as orthophosphate, sulphate citrate, or bicarbonate, can not replace chloride in the uptake process (Lingjærde 1971 b) Nitrite was found to have a more complex effect on the uptake than chloride, and it was suggested that nitrite besides being able to replace chloride in the "anionic site' of the 5HT carrier also acts on an 'activating site", which may be situated on the carrier itself or on some other part of the plasma membrane (Lingjærde 1971 b) However since nitrite is not normally present in plasma in appreciable amounts, it cannot be a physiological activator of 5HT uptake in platelets The hypothetical physiological activator (or activators) presumably has anionic character, like nitrite Phosphate or bicarbonate would seem to be likely candidates, but they do not have the same effects as nitrite on the 5HT uptake (Lingjærde 1971 a)

Further experiments have revealed that different monocarboxylic acids, such as acetate, lactate, and pyruvate, have a stimulatory effect on 5HT uptake when present together with chloride, whereas they have no significant effect in the absence of chloride The purpose of the present study was to characterize the effects of acetate and other monocarboxylic acids on the uptake of 5HT in platelets, and to compare these effects with those previously described for nitrite (Lingjærde 1971 b). For practical reasons, the investigations were mainly concentrated on the effects of acetate, whereas the effects of other monocarboxylic acids have been subjected only to some preliminary studies

Materials and methods

Platelets from healthy persons were isolated by differential centrifugation resuspended in a simple phosphate—buffered medium, and incubated at 37° C with ^{14}C-5HT, the net uptake of ^{14}C-5HT being subsequently assayed by liquid scintillation spectrometry Further details concerning materials and methods are described elsewhere (Lingjærde 1971 a, 1971 b)

Results

1. *Effects of acetate in the presence of chloride*

Fig 1 shows the effects of increasing concentrations of acetate in the presence of 75 mM chloride, at pH 7 3 and 6 2 At pH 7 3 acetate had a stimulatory effect which reached a maximum at about 10—20 mM The stimulatory effect was some-

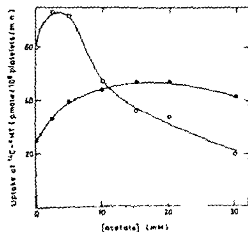


Fig 1

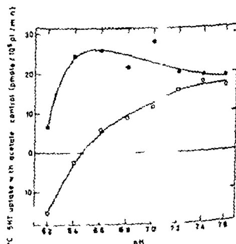


Fig 2

Fig 1 The uptake of ^{14}C-5HT versus concentration of acetate, at pH 7 3 (●) and 6 2 (○) The mixtures were incubated with 1 μM ^{14}C 5HT for 5 min Each point represents the mean of two parallel samples

Fig 2 The effects on ^{14}C-5HT uptake of 10 mM (●) and 30 mM (○) acetate at varying pH The mixtures were incubated with 1 μM ^{14}C 5HT for 5 min The effects are shown as difference between uptake in the presence and absence of acetate All samples were run in duplicate

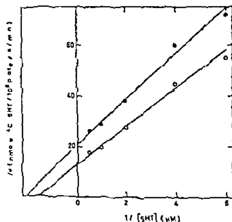


Fig 3

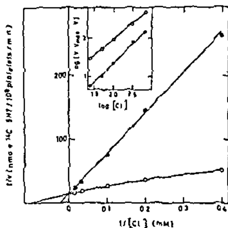


Fig 4

Fig 3 Double reciprocal plots of ^{14}C 5HT uptake versus ^{14}C 5HT concentration at pH 7 3, in the presence (○) and absence (●) of 10 mM acetate The mixtures were incubated with ^{14}C-5HT for 4 min Each point represents the mean of two parallel samples

Fig 4 Main figure Double reciprocal plots of ^{14}C 5HT uptake versus chloride concentration at pH 7 3, in the presence (○) and absence (●) of 10 mM acetate The mixtures were incubated with 1 μM ^{14}C 5HT for 5 min Each point represents the mean of two parallel samples Inserted figure Hill plots of the same data

what reduced at higher concentrations The maximal stimulatory effect of acetate in this experiment was about 80 % over the control value, whereas in different analogous experiments it varied from 10 to 90 %, probably mainly due to individual differences

At pH 6 2 the effect of acetate was quite different At low concentrations, a moderate stimulation is seen, whereas at higher concentrations there was a marked inhibitory effect on the 5HT uptake

The relationship between pH and the effects of high and low concentrations of acetate is shown in more detail in Fig 2 It is seen that 10 mM acetate had little stimulatory effect at pH 6 2 The stimulation was increased at higher pH, up to about pH 6 6, and was slightly decreased at still higher pH values High concentration of acetate (30 mM) had an inhibitory effect at pH 6 2, with a gradual change to a marked stimulatory effect at higher pH values

In a control experiment it was found that the stimulatory effect of 15 and 30 mM acetate at pH 7 3, as well as the inhibitory effect of 15 and 30 mM acetate at pH 6 0, were not influenced by preincubation with acetate for up to about 30 min Thus, these effects of acetate seem to be 'immediate', and not time-dependent effects

The effect of 10 mM acetate on the kinetics of the uptake at pH 7 3 is shown in double reciprocal plots in Fig 3 The plots are linear and acetate gives about the same percentage increase in $V_{max\ 5HT}$ (i e, the rate of uptake at infinite concentrations of 5HT) and apparent $K_{m\ 5HT}$ (i e the concentration of 5HT

TABLE I Temperature effect on acetate stimulation

Temp (° C)	a Without acetate	b With acetate	b—a
0	2 41±0 06	2 12±0 04	—0 3
12	4 33±0 18	3 92±0 20	—0 4
25	10 7 ±0 52	18 0 ±1 95	7 3
37	33 0 ±0 84	51 2 ±1 62	18 2

The platelets were resuspended in the standard medium with and without 10 mM acetate added The mixtures were preincubated without 5HT for 5 min and thereafter incubated with 1 μM ^{14}C 5HT for 5 min The net uptake is given as mean of three parallel samples ± s e m (a n t pmole ^{14}C-5HT/10^8 platelets/min)

which gives half maximal uptake rate) A corresponding but opposite change of the kinetic parameters was found when 5HT uptake was plotted against 5HT concentration at 0 mM, 15 mM and 30 mM acetate at pH 6 2 $V_{max\ HT}$ and apparent $K_{m\ (HT)}$ underwent a similar relative decrease so that the effect on the slope of the double reciprocal plots was very small Thus the inhibitory effect of acetate at pH 6 2 is best described as uncompetitive as to 5HT

The effect of temperature on the stimulatory effect of acetate at pH 7 3 is shown in Table I Like the uptake without acetate the stimulation by acetate is seen to be highly dependent on temperature At 12° C there is a small but significant uptake in the controls but no significant stimulatory effect of acetate Between 25 and 37° C the acetate stimulation has a Q_{10} of about 2, [illegible] temperature dependency is of a magnitude which is common for enzymatic [illegible]

Fig 4 shows the effect of increasing concentrations [illegible] with and without 10 mM acetate added In accordance with previou[s] [illegible] chloride effect (without acetate) is obeying simple saturation kinet[ics] [illegible] 1971 a) with an interaction coefficient according to Hill plots o[f] [illegible]) In the presence of acetate $V_{max\ Cl}$ is unchange[d] [illegible] is markedly decreased Thus acetate seems to have t[he] [illegible] affinity of the carrier for chloride

The double reciprocal plot in the presence o[f] [illegible] upwards) and the Hill plot gives an interacti[on] [illegible] 10 1 02 without acetate This might indicate that [illegible] of negative cooperativity in the presence of ac[etate] [illegible] tion from simple saturation kinetics requires [illegible]

2 *Interactions between acetate nitrite, and po[tassium]* [illegible]

The effect of increasing concentrations of nitrite [illegible] mM acetate is shown in Fig 5 As has been [illegible] a sigmoidal uptake curve with an interaction [coefficient of] about 2 (Lingjærde 1971 b) In the presence of [illegible] markedly reduced giving an interaction coeffic[ient] [illegible]

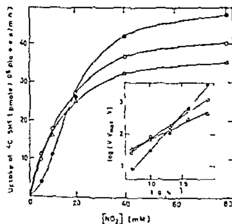


Fig 5

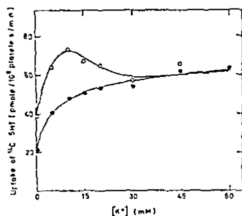


Fig 6

Fig 5 Main figure The uptake of ^{14}C 5HT versus nitrite concentration, in the presence of 0 mM (●) 10 mM (○), and 30 mM (Δ) acetate The mixtures were incubated with 1 μM ^{14}C-5HT for 5 min Each point represents the mean of two parallel samples Inserted figure Hill plots of the same data (Note that the units on the abscissa are 2 × those on the ordinate)

Fig 6 The uptake of ^{14}C 5HT versus potassium concentration, at pH 7.3, in the presence (○) and absence (●) of 20 mM acetate Potassium was added as the sulphate so that the concentration of both chloride and sodium remained constant The mixtures were incubated with 1 μM ^{14}C-5HT for 4 min Each point represents the mean of two parallel samples

the nitrite curve is completely "michaelized , showing simple saturation kinetics and an interaction coefficient of 0 98 Double reciprocal plots of these data show that $V_{max(NO_2^-)}$ is not significantly altered by acetate (not shown in Fig 5, but is also seen from Fig 7)

It has been demonstrated previously that potassium has a stimulatory effect on 5HT uptake in platelets (Born and Gillson 1959, Weissbach and Redfield 1960, Rysanek *et al* 1967, Lingjærde, unpublished) To investigate the possible relationship between the stimulatory effects of potassium and acetate platelets were incubated with increasing concentrations of potassium with and without 20 mM acetate present The result is shown in Fig 6 It appears that the stimulatory effect of acetate is highly dependent on the potassium concentration There is a significant effect in the absence of potassium the effect is maximal between 5 and 10 mM potassium, whereas it is totally abolished when potassium concentration is higher than about 30 mM In another experiment it was found that the same is true also for 10 and 30 mM acetate It is to be noted that in this medium with chloride as the active anion and without acetate there is no tendency for higher concentrations of potassium (up to 60 mM) to reduce the uptake

This experiment may be intepreted in two different ways (1) Potassium stimulates by the same mechanism as does acetate and therefore acetate has no additional effect when the concentration of potassium is high, or (2) potassium stimulates by a different mechanism but at the same time higher concentrations of potassium

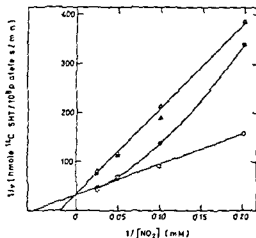


Fig 7

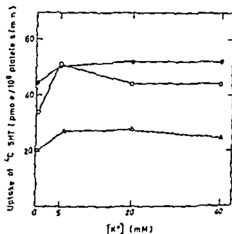


Fig 8

Fig ~ ... us nitrite concentration in the presence of th... us 20 mM acetate (O) 40 mM potas sium ... (▲ only three points) The mixtures were ... ch point represents the mean of two parallel samples

Fig 8 The uptake of ^{14}C 5HT versus potassium concentration at pH 6 2 in the presence of 0 mM (●) 15 mM (O), and 30 mM (▲) acetate Potassium was added as KCl with a corresponding reduction of NaCl The mixtures were incubated with 1 μM ^{14}C 5HT for 4 min Each point represents the mean of two parellel samples

lock the action of acetate It will be seen from the next experiments that the latter possibility is the more likely one

In the next experiment, platelets were incubated with increasing concentrations of nitrite in the presence of (1) the usual 5 mM potassium (controls) (2) 5 mM potassium and 20 mM acetate (3) 40 mM potassium and (4) 40 mM potassium and 20 mM acetate The result is shown in double reciprocal plots in Fig 7 It is seen that both acetate and a high concentration of potassium change the sigmoidal nitrite curve to a hyperbolic saturation curve and neither has any significant effect on $V_{max\ NO_2}$ However the effects of acetate and high concentration of potassium are still quite different Whereas acetate acts as a stimulator at low concentrations of nitrite potassium on the contrary acts as an inhibitor Moreover in the presence of acetate and 5 mM potassium apparent $K_{m\ NO_2}$ is less than half of that found in the presence of 40 mM potassium In the presence of both acetate and 40 mM potassium the uptake is similar to that obtained with 40 mM potassium without acetate Thus this experiment shows that in a nitrite medium 40 mM potassium blocks the stimulatory effect of acetate and inhibits the effect of nitrite For further discussion see below

Fig 8 shows the effect of potassium concentration on the inhibitory effect of 15 and 30 mM acetate at pH 6 2 The inhibitory effect of acetate was not blocked by high concentrations of potassium indicating that this effect is quite independent of the stimulatory effect

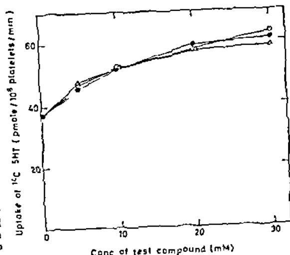


Fig 9 The uptake of ^{14}C-5HT versus increasing concentrations of acetate (●), l-d lactate (○), and pyruvate (Δ) The test compounds were added as the sodium salts, in exchange for Na_2SO_4 The mixtures were incubated with 1 μM ^{14}C 5HT for 5 min Each point represents the mean of two parallel samples

3 *Effects of acetate in the presence of other anions*

In one experiment, the effect of 10 mM acetate in the presence of different anions (in the concentration of 75 mM), at pH 7 3, was investigated Acetate had a significant stimulatory effect in the presence of chloride, bromide, and iodide, but no stimulatory effect in the presence of nitrite or nitrate

4 *Effect of acetate on 5HT outflux*

Acetate (10 or 30 mM) had no appreciable effect on 5HT outflux rate, whether at pH 6 2 or pH 7 3, making the present determinations of net flux a reliable measure of influx (see also Lingjærde 1971 b)

5 *Effects of other monocarboxylic acids*

The effects of acetate at pH 7 3 is not specific for this monocarboxylic acid Table II shows that formate, which has a small effect even in absence of chloride (Lingjærde 1971 b), exerts a stimulatory effect of the same magnitude as that of acetate Propionate and butyrate in the concentration of 10 mM also exert a significant stimulatory effect, although less than acetate However raising the concentration of propionate and butyrate to 30 mM gave a marked inhibitory effect analogous to the effect of acetate at low pH (see Fig 1)

It was also found that lactate and pyruvate stimulate the uptake at pH 7 3 Thus, in one experiment (Fig 9) platelets were incubated in a standard chloride medium with varying concentrations of acetate l-d lactate, or pyruvate The effects of these compounds were remarkably similar i e they all exerted a stimulatory effect at this physiological pH, with a maximum effect at about 20 mM

Discussion

Previous studies have indicated that the 5HT carrier in the platelet plasma membrane has an "anionic site", to which a small anion (usually chloride) has to be bound if the translocation of the carrier, from being "open" outwards to being "open" inwards, is to take place (Lingjærde 1971 a) Furthermore the complex effects of nitrite have indicated that this anion not only can replace chloride in the anionic site, but also exerts a "specific activating effect" on the 5HT uptake by interacting with a hypothetical 'activating site', which is probably also situated on the carrier (Lingjærde 1971 b) The main effect of a specific activator would be to increase the affinity of the anionic site for chloride (or other small univalent anions)

In contrast to nitrite, acetate has practically no effect on 5HT uptake in the absence of chloride (or other small anions), and it is therefore suggested that acetate has no direct effect on the anionic site, probably because this anion is too large (see Lingjærde 1971 b) However, acetate seems to react with the 'activating site' in the same way as does nitrite, for the following reasons

First the main effect of acetate (at pH 7 3) is to increase the affinity of the anionic site for small anions in this case chloride (Fig 4) This is analogous with the previously reported effect of nitrite (Lingjærde 1971 b)

Second in the presence of chloride acetate gives about the same percentage increase of $K_{m\ 5HT}$ and $V_{max\ 5HT}$ (Fig 3) which is also analogous with the effect of nitrite in the presence of relatively high concentrations of chloride (It may be noted that the effect on $V_{max\ 5HT}$ does not necessarily mean that acetate or nitrite increase the rate of translocation of the carrier since this effect can be explained as due to a higher affinity of the carrier for chloride which in these experiments was present in a fixed concentration)

Third the interaction between acetate and nitrite is in agreement with the assumption that they both have an effect on the same activating site In the presence of acetate (i e with the carrier already in the activated state nitrite gives a simple saturation curve with about the same $V_{max\ NO_2^-}$ as found without acetate (Fig 7) Thus the stimulatory effects of acetate and nitrite in optimal concentrations, are non additive

Fourth the stimulatory effects of acetate and nitrite are both inhibited by high concentrations of potassium (Fig 6 and 7 Since nitrite presumably reacts with both the anionic site and the activating site whereas acetate reacts with only the latter, a blocking of the activating site would be expected to abolish the effect of acetate completely and abolish the effect of nitrite only partially This is actually what was found It thus seems reasonable to assume that the upper curve in Fig 7 (i.e., the nitrite curve in the presence of 40 mM potassium) reflects the effect of nitrite solely on the anionic site

As shown in Fig 7 potassium acts as a competitive inhibitor of specific activators However, it seems rather unlikely that potassium a cation can be bound

Table II. Effects of different monocarboxylic acids on uptake of ^{14}C-5HT, at pH 7.3

Test compound	conc. (mM)	^{14}C-5HT uptake
control		40.8 ± 1.6
formate	10	63.0 ± 1.5
	30	67.3 ± 1.4
acetate	10	61.3 ± 1.7
	30	64.6 ± 1.5
propionate	10	59.7 ± 1.2
	30	17.0 ± 0.6
butyrate	10	53.7 ± 2.3
	30	25.6 ± 1.4

The mixtures were preincubated without 5HT for 5 min and thereafter incubated with 1 μM ^{14}C-5HT for 5 min. The results are given as mean of three parallel samples ± s.e.m. (unit: pmole ^{14}C-5HT/10^8 platelets/min)

to the same site as the anionic activators. Probably, high concentrations of potassium reduce the affinity of the activating site for acetate and nitrite by an indirect effect.

As mentioned in Results, potassium has previously been shown to have a stimulatory effect of its own on 5HT uptake in platelets. This is also seen from Fig. 6 and 8 in this paper. From the results discussed above it must be assumed that the stimulatory effect of potassium is independent of the stimulatory effect of acetate and nitrite. Thus, high concentrations of potassium block the stimulation exerted by acetate and nitrite without reduction of the stimulation by potassium itself.

At pH 6.2 acetate has a rather marked inhibitory effect on the 5HT uptake, and also at pH 7.3 there is a tendency towards an inhibitory effect at higher concentrations. Since this inhibitory effect is unaffected by increasing concentrations of potassium (Fig. 8), it is assumed to be independent of the stimulatory effect, i.e. it is not brought about by an effect on the activating site. The inhibition is best described as uncompetitive. This would mean that the inhibitor reacts with a form of the carrier which is (1) different from and (2) irreversibly connected with the variable substrate, *i.e.*, 5HT (according to the enzyme kinetic models of *Cleland* 1963). The inhibitory effect of acetate is kinetically of the same type as the inhibition by the drugs ergotamine and dihydroergotamine (although their inhibitory effect was described as non-competitive rather than uncompetitive (Lingjærde 1970)) as well as medazepam (Lingjærde, unpublished), but it remains to be seen whether these compounds share a common mechanism of action.

Acetate stimulates not only in the presence of chloride but also in the presence of bromide and iodide. Like chloride, bromide and iodide would therefore seem to be without an effect on the activating site. However, it was previously shown that increasing concentrations of bromide give a slightly sigmoidal 5HT uptake curve (Lingjærde 1971 b), which would seem to indicate that bromide has a weak

TABLE I Personal data of research subjects and intensity of work employed in negative work

Subject	Age	Height (cm)	Weight (kg)	Max V_{O_2} (l/min)	Work Intensity (Watts)
HF	29	181 6	73 0	3 80	210
LV	20	177 1	67 0	3 95	200
NR	22	184 2	81 0	3 99	230

50 min at least 4 times per week for 3—5 weeks The work intensities are given in Table I V_{O_2} was determined for short periods at 10—15 min intervals Expired gas was collected in Douglas bags, measured for volume in a balanced spirometer, and analysed for O_2 and CO_2 content according to Scholander (1947) Heart rate was counted by EKG or auscultation throughout each exercise period The room temperature was kept at 20±1° C

Results

The V_{O_2} from selected experiments during the training periods are presented in Fig 1 It was observed in all subjects that, prior to training, the V_{O_2} increased considerably during the period of exercise This increase averaged over 300 ml/min or more than 25 % from the 10th to the last min of exercise An increase of this magnitude is never observed during corresponding periods of submaximal positive work

With repeated bouts of exercise both the V_{O_2} after 10 min of exercise and the increase observed during the remainder of the exercise periods diminished There was a difference in the speed of response of the three subjects to training, most

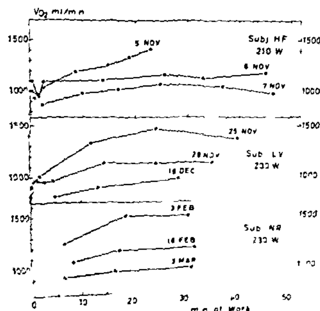


Fig 1 Oxygen consumption plotted against time during long lasting exercise with negative work Dates of experiments are indicated

probably due to differences in the training programs The first subject (HF) had trained for over a month at various negative work intensities before all training was performed at a high negative work intensity (numerically 210 W) Already after a few days of such training, a dramatic lowering in V_{O_2} had taken place The other two subjects (LV and NR) performed all of their training at high numerical work intensities (200 and 230 W respectively) and the same lowering in V_{O_2} took place but occurred over a longer period of time (see Fig 1)

Heart rate was closely related to V_{O_2} and increased or decreased in each subject in the same direction as the changes in V_{O_2}, regardless of time in all experiments Little or no change was observed in this relationship as a result of training

An additional observation related to the training effect was that in the early experiments where the V_{O_2} demonstrated the continuous and large increase, the subjects sweated profusely After the V_{O_2} had decreased to a fairly constant level the subjects showed no more than a nominal amount of sweating

Two of the subjects were reexamined 3—4 months after the training experiments were terminated The same work intensities as indicated in Fig 1 were employed At 7 17 and 27 min of work, the V_{O_2} was as follows 944 1203, and 1228 ml/min for subject LV and 1058, 1356, and 1523 ml/min for subject HF The return to the higher values and the reappearance of the large increase in V_{O_2} during the work period resembled the condition partway through the training for subject LV and the pre training state for subject HF Heart rate increases corresponded to increases in V_{O_2} profuse sweating similar to that observed in a pretraining state was again observed

Discussion

The decrease in energy release after the training period as calculated from V_{O_2} was comparable in magnitude to the gravitational work performed on the body through the bicycle The differences between the highest V_{O_2} values recorded for the 3 subjects in an untrained state (where large increases were observed) and the terminal V_{O_2} values after training corresponded in caloric equivalents (using 4 9 kcal/l O_2) to 2 6 (HF), 2 4 (LV) and 2 5 (NR) kcal/min The caloric equivalents of the energy absorbed, were 3 0 (HF), 2 9 (LV) and 3 3 (NR) kcal/min In other words the energy delivered by aerobic processes decreased by an amount approximating the gravitational work being absorbed during the exercise

Abbott and co-workers (1950, 1951 a, 1951 b) have found that work performed on stimulated frog muscle by stretching could not be accounted for as heat or elastic potential energy They hypothesized that the chemical reactions occurring during development of tension were "driven backwards" during the stretching Subsequent work by Aubert (1954, 1956) and by Hill and Howarth (1960) further supported the contention that a large portion of the energy from the eccentric contractions was absorbed Experiments by Curtin *et al* (1970), Marechal (1964) and Wilkie (1968) have shown that the biochemical relationships are distinctly different

TABLE I Personal data of research subjects and intensity of work employed in negative work

Subject	Age	Height (cm)	Weight (kg)	Max $\dot{V}_{O_2}$ (l/min)	Work Intensity (Watts)
HF	29	181 6	73 0	3 80	210
LV	20	177 1	67 0	3 95	200
NR	22	184 2	81 0	3 99	230

50 min at least 4 times per week for 3—5 weeks The work intensities are given in Table I $\dot{V}_{O_2}$ was determined for short periods at 10—15 min intervals Expired gas was collected in Douglas bags measured for volume in a balanced spirometer, and analysed for O_2 and CO_2 content according to Scholander (1947) Heart rate was counted by EKG or auscultation throughout each exercise period The room temperature was kept at 20±1° C

Results

The $\dot{V}_{O_2}$ from selected experiments during the training periods are presented in Fig 1 It was observed in all subjects that, prior to training the $\dot{V}_{O_2}$ increased considerably during the period of exercise This increase averaged over 300 ml/min or more than 25 % from the 10th to the last min of exercise An increase of this magnitude is never observed during corresponding periods of submaximal positive work

With repeated bouts of exercise both the $\dot{V}_{O_2}$ after 10 min of exercise and the increase observed during the remainder of the exercise periods diminished There was a difference in the speed of response of the three subjects to training most

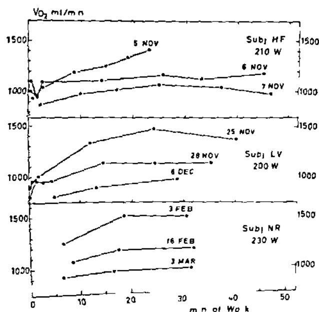


Fig 1 Oxygen consumption plotted against time during long lasting exercise with negative work Dates of experiments are indicated

HILL A V and J V HOWARTH The reversal of chemical reactions in contracting muscle

with concentric and

triphosphate durant

SCHOLANDER P F, Analyzer for accurate estimation of respiratory gases in one half cubic centimetre samples *J biol Chem* 1947
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Acta physiol scand 1971. 83. 324—334

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The Metabolism of Monosaccharides in Isolated Rat Mast Cells and its Influence on Histamine Release Induced by Adenosine-5'-Triphosphate.

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Abstract

DIAMANT B and C PETERSON *The metabolism of monosaccharides in isolated rat mast cells and its influence on histamine release induced by adenosine 5' triphosphate* Acta physiol scand 1971 83 324—334

Histamine release from isolated rat mast cells induced by extracellularly applied ATP in the presence of calcium was enhanced by D(+)glucose and D(+)mannose but not by D(−) fructose or D(+)galactose (0 6 mM of each) Only those monosaccharides which enhanced the [illegible] path way [illegible] (1 [illegible]
Antimycin A (10 [illegible] M) completely inhibited ATP induced histamine release [illegible] the presence of glucose pyruvate or lactate Iodoacetate (10^{-4} M) blocked the enhancing effect of glucose but not that of pyruvate or lactate In the presence of glucose (0 6 mM) or mannose (0 6 mM) the accumulation of lactate in suspensions of rat mast cells was enhanced by antimycin A (10^{-7} M) and blocked by iodoacetate (10^{-4} M) In the absence of glycolytic substrates and metabolic inhibitors no accumulation of lactate was noted whereas in the presence of antimycin A (10^{-7} M) a slight but statistically significant accumulation occurred

Histamine release from isolated rat mast cells is induced by extracellularly applied adenosine 5'-triphosphate (ATP) in the presence of Ca (Diamant and Kruger 1967, Sugiyama and Yamasaki 1969) The mechanism for the ATP induced histamine release differs in various respects from that of other agents studied e g compound 48/80 (Diamant 1969) However a common feature for histamine release induced by ATP and 48/80 is the energy dependence Whereas inhibition of 48/80-induced histamine release exerted by inhibitors of oxidative metabolism could be fully reversed by the presence of glucose (Diamant and Uvnas 1961) this hexose did not markedly restore ATP-induced histamine release under similar conditions (Diamant and Kruger 1967)

The available data suggested that glycolytic energy production sufficed to furnish energy to the histamine releasing process when induced by compound 48/80 whereas histamine release induced by extracellular ATP required a functioning oxidative phosphorylation (Diamant 1969)

Furthermore it was shown that the addition of glucose to isolated rat mast cells incubated in an aerobic milieu enhanced histamine release induced by extracellularly applied ATP (Diamant and Peterson 1970) Glucose could not be demonstrated to enhance histamine release induced by compound 48/80 under identical experimental conditions In the presence of 0 3—0 5 mM of glucose ATP induced histamine release increased to 1 5—2 times of that observed in the absence of glucose Glucose did not influence the initial lag period but after histamine release had started it occurred at a higher velocity in the presence of glucose than in its absence

In an earlier investigation (Diamant and Uvnas 1961) it was shown that in the presence of glucose anoxia was ineffective to inhibit histamine release caused by compound 48/80 *from minced lung tissue whereas galactose or fructose did not* influence the inhibition The metabolism of these hexoses in isolated rat mast cells was studied by Chakravarty (1968) by the use of labelled monosaccharides As judged from the production of ^{14}CO all three sugars were taken up by the mast cells and metabolized by the oxidative pathway

Thus there seems to be a discrepancy between the metabolic utilization of the hexoses and their effectiveness to counteract a block of oxidative metabolism (anoxia) as judged from their effect on histamine release induced by compound 48/80

In the present investigation the enhancing effect of glucose and other monosaccharides on ATP induced histamine release from isolated rat mast cells in an aerobic milieu was correlated to the metabolism of the monosaccharides by the cells As an indication of metabolism lactate was determined in mast cell suspensions incubated in the presence and absence of sugars and metabolic inhibitors

The results show that there is a correlation between the metabolism of the sugars as judged from their conversion to lactic acid and their enhancing effect on histamine release induced by ATP in an aerobic milieu

Methods and Materials

Isolation of rat mast cells

Rat mast cells were isolated from the peritoneal and pleural cavities of male Sprague Dawley rats (weight 350—400 g) in general following the method given by Uvnas and Thon 1959 Glucose was however omitted from all solutions In each experiment cells from 1—8 rats were used

After separation of the cells by Ficoll density gradient centrifugation the mast cells were washed 3 times with a balanced salt solution containing NaCl 145 mM KCl [illegible] 7 mM $CaCl_2$ (0 7 mM) and [illegible] buffered pH [illegible] 0 with 10 (v/v) Son[illegible] tion [illegible] ly suspended in the buffered salt sol mer[illegible] 1×10^6 cells ml in different e[illegible]

Incubation procedures

All incubations of mast cells were carried out at +37° C and at pH 7 0 When necessary (after the addition of lactic acid) the pH was adjusted to 7 0 with NaOH All experiments were run in duplicate samples and the mean values were used for calculations

Experiments concerning histamine release

1 8—4 8 μl of the mast cell suspension were added to open test tubes (volume 10 ml) containing 2 ml of the buffered salt solution (see above), giving a cell concentration varying between 2 6—4 9×10³ cells/ml Unless otherwise stated the mast cells were preincubated for 10 min in the presence or absence of metabolic substrates and inhibitors before ATP (3.5—3 8×10⁻⁵ M) was added and thereafter the incubation was continued for 15 min Incubation was stopped by placing the tubes in ice cold water The tubes were then centrifuged (350×g) for 10 min at +4° C The supernatants were carefully decanted and 2 ml of distilled water were added to the residues in order to release the remaining histamine Histamine was determined in the supernatants and the re [illegible] Shore B rkhalter and Cohn (1959) omitting the extr [illegible] was then calculated as percentage of [illegible] histamine release averaged 4 0±0 31 [illegible] release of [illegible]

Antimyc [illegible] final concentration of ethanol d[illegible] [illegible]riments with antimycin A all incubations were made to contain 0 00 [illegible] of ethanol [illegible] concentration of ethanol did not affect ATP induced histamine release from rat mast cells or histamine determination

[illegible] es (volume 3 ml) giving a final [illegible] The incubation was started by [illegible] controls an equivalent volume [illegible] after 15 min by adding NaOH (final concentration 0 02 M) immediately before heating the tubes at +60° C for 10 min Time zero in the figures denotes samples which were heated at +60° C for 10 min in the presence of 0 02 M NaOH without incubation at 37° C

Another experimental procedure was used when investigating the time course for lactate accumulation in samples containing isolated rat mast cells in the presence of glucose with and without antimycin A 700 μl of the mast cell su[illegible] [illegible] 3 1 and 4 6×10⁶ cells/ml respectively) were incubated f[illegible] mycin A before the addition of glucose Imme[illegible] various times thereafter samples (100 μl) were [illegible] were heated at 60° C for 10 min Samples containing antimycin A also contained e[illegible] (0 12 % v/v) This concentration of ethanol did not influence the accumulation or determination of lactate

Blanks and standards with known concentrations of lactate were carried through the incubation procedure

Lactate was measured according to the fluorometric method given by Rovainen Lowry and Passonneau (1969) 75 μl of samples standards or blanks were added to 1 ml of a reagent solution containing β NAD (1 mM) and beef heart lactic dehydrogenase (9 μg/ml) in carbonate buffer (0 1 M pH 9 7) The half time of the reaction was <1 min The lactate concentrations of the samples were expressed as mmoles lactate/kg mast cells (dry weight) The dry weight of the cells was calculated from the cell count and the dry weight 0 5×10⁻¹⁴ kg/mast cell (Diamant and Lowry 1966)

Materials

Human serum albumin was kindly supplied by AB Kabi (Stockholm Sweden) Ficoll was obtained from AB Pharmacia (Uppsala Sweden) D(+)glucose was purchased from Mallinchrodt Chemical Works (USA) L glucose D(+)mannose D(+)galactose βD(—)fructose D(—)ribose 2 deoxy D ribose antimycin A β NAD (yeast) pyruvate (sodium salt) adenosine 5 triphosphate (disodium salt) were [illegible] (St. Louis USA) and L(+)lactic acid from Boehri[illegible] Stock solutions of ATP (neutralized with NaOH) [illegible] enzymatic spectrophotometric methods used in the laboratory of dr [illegible] [illegible]ouis USA) Between use stock solutions were stored at −20° C Beef heart lactate dehydrogenase was purchased from Worthington Biochemical Corp (USA) iodoacetic acid from Merck AG (Darmstadt, Germany) and phtaldialdehyd from Fluka AG (Switzerland) Enzymatic fluorometric analysis of the preparation of mannose revealed that it contained 0 1 % of D(+) glucose

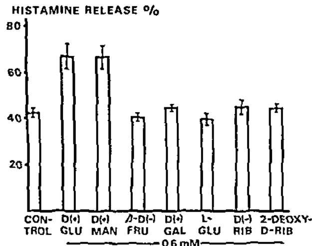


Fig 1 Histamine release from isolated rat mast cells induced by ATP ($3 8 \times 10^{-5}$ M) in the presence of D(+)glucose, D(+)-mannose D(−)fructose D(+) galactose L-glucose, D(−)ribose and 2 deoxy D ribose (0 6 mM of each) and in the absence of monosaccharides. The mast cells were preincubated with and without the monosaccharides for 10 min before the addition of ATP and the incubation was then continued for another 15 min 4 expts were performed each in duplicate samples Mean values ± S E are shown

Results

D(+)mannose (0 6 mM) enhanced ATP induced histamine release to the same extent as D(+)glucose (0 6 mM) (Fig 1) The effect of both hexoses was statistically significant ($p < 0 01$) On the other hand, in the presence of D(−)fructose, D(+)galactose, L-glucose, D(−)ribose and 2 deoxy-D ribose (0 6 mM of each) histamine release did not significantly differ from controls incubated in the absence of monosaccharides

Lactate was found to accumulate in mast cell suspensions incubated with the same monosaccharides as were effective in potentiating ATP induced histamine release (Fig 2) Thus, the lactate concentration in mast cell suspensions incubated for 15 min at +37° C in the presence of D(+)glucose (0 6 mM) or D(+)mannose (0 6 mM) was significantly higher ($p < 0 01$) than in samples incubated in the

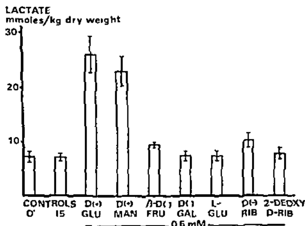


Fig 2 Lactate contents in suspensions of isolated rat mast cells incubated for 15 min in the presence of D(+)glucose D(+) mannose D(−)fructose D(+) galactose L-glucose, D(−)ribose and 2-deoxy D ribose (0 6 mM of each) and in the absence of monosaccharides 4 expts were performed each in duplicate samples Mean values ± S E are shown

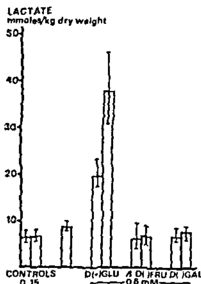


Fig 3

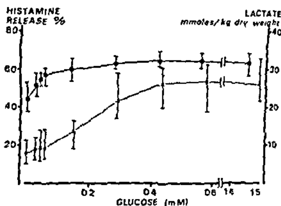


Fig 4

Fig 3 Lactate content in suspensions of isolated rat mast cells incubated for 15 min with D(+)glucose, D(−)fructose and D(+)galactose (0 6 mM of each) and without monosaccharides in the presence (dotted bars) and absence (open bars) of antimycin A 1 3 × 10^{-7} M) 3 expts were performed, each in duplicate samples Mean values and range are shown

Fig 4 The [illegible] release ([illegible] pensions of [illegible] ATP and gl[illegible]
All samples were incubated for 15 min 3 expts were performed, each in duplicate samples Mean values and range are shown

absence of monosaccharides or in the presence of those sugars which did not enhance histamine release

However, the possibility still existed that those monosaccharides, which did not give rise to lactate accumulation under these conditions were metabolized to pyruvate at a slow rate which did not exceed the capacity of the cells to further metabolize pyruvate through the oxidative pathway Therefore the influence of D(+)-glucose, fructose and galactose on lactate accumulation in mast cell suspensions were investigated in the presence of an inhibitor of oxidative phosphorylation (antimycin A, 10^{-7} M) (Fig 3) The results show that neither fructose nor galactose caused an accumulation of lactate in the presence of antimycin A that differed from the values found in absence of the sugars In contrast, in the presence of antimycin A glucose induced an additional accumulation of lactate of about 100 %

In mast cell suspensions containing antimycin A but no monosaccharides, a slight but statistically significant accumulation of lactate was observed after incubation at +37° C for 15 min (Table I) In contrast in the absence of inhibitor the lactate concentration was unaffected

TABLE I. Lactate content in samples of isolated rat mast cells before and after incubation for 15 min in the presence and absence of antimycin A (1.3 × 10⁻⁶ M). No monosaccharides were added. [illegible] experiments were performed, each in duplicate samples.

Lactate	Before incubation	After incubation for 15 min	After incubation for 15 min in the presence of antimycin A
nmoles/mg dry weight, mean value ± S.E.	5.6 ± 0.3	5.8 ± 0.8 NS	8.3 ± 0.7 p < 0.0[illegible]

The lactate accumulation in the cell suspensions was found to be dependent on the concentration of glucose (Fig. 4). Whereas histamine release induced by ATP from isolated rat mast cells was clearly enhanced already in the presence of 0.0[illegible] mM of glucose, lactate accumulation was not ascertainable until the concentration of glucose was raised above 0.06 mM. Lactate production then increased with increasing concentrations of glucose up to 0.4 mM without apparent increase of histamine release. Further increase of the glucose concentration up to 1.5 mM did not result in increased lactate accumulation or histamine release.

Since glucose and mannose both caused accumulation of lactate and enhancement of ATP-induced histamine release in suspensions of isolated rat mast cells, it was of interest to investigate whether the glycolytic end products pyruvate and lactate might also enhance ATP induced histamine release. As shown in Fig. [illegible] both pyruvate (1 mM) and lactate (1 mM) potentiated ATP induced histamine release ($p < 0.005$ and $p < 0.0[illegible]$, respectively). Antimycin A (10⁻⁶ M) totally blocked ATP induced histamine release irrespectively of the presence of glucose, pyruvate or lactate. Without the addition of metabolic substrates, iodoacetate (10⁻⁴ M) partially inhibited ATP induced histamine release ($p < 0.0[illegible]$). In the presence of iodoacetate, pyruvate (1 mM) and lactate (1 mM) still enhanced the histamine release induced by ATP ($p < 0.001$), whereas no effect was noted for glucose (0.6 mM).

The effect of antimycin A (10⁻⁶ M) and iodoacetate (10⁻⁴ M) on lactate accumulation in rat mast cell suspensions incubated in the presence and absence of glucose and mannose (0.6 mM of each) is shown in Fig. [illegible]. In the presence of antimycin A, mannose as well as glucose gave rise to an additional accumulation of lactate as compared to cell suspensions incubated with sugars in the absence of the inhibitor ($p < 0.01$). In the presence of iodoacetate no lactate accumulation was found in the presence of glucose or mannose.

The time course for the lactate accumulation in suspensions of rat mast cells incubated with glucose (0.6 mM) in the presence and absence of antimycin A (10⁻⁶ M) is demonstrated in Fig. 7. Lactate increased linearly with time in both cases. The rate of accumulation was about twice as high in the presence of antimycin A as in its absence. The lactate content of [illegible] incubated without glucose at 37° C for 15 min was used as control in these experiments.

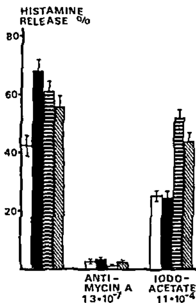


Fig 5

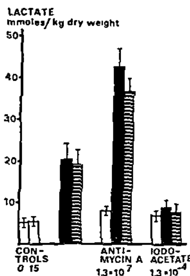


Fig 6

Fig 5 The effect of antimycin A (1.3×10^{-7} M) and iodoacetate (1.1×10^{-4} M) on the histamine release from isolated rat mast cells induced by ATP (3.7×10^{-6} M) in the presence of glucose (0.6 mM) (filled bars) pyruvate (1 mM) (horizontally striped bars) and lactate (1 mM) (angularly striped bars) and in the absence of added metabolic substrates (open bars) The cells were preincubated for 10 min in the presence or absence of substrates and inhibitors before the addition of ATP The incubation was then continued for another 15 min 7 expts were performed each in duplicate samples 5 of the expts included the effect of antimycin A and 6 expts that of iodoacetate Mean values ± S E are shown

Fig 6 The effect of antimycin A (1.3×10^{-7} M) and iodoacetate (1.3×10^{-4} M) on the lactate content in suspensions of isolated rat mast cells incubated in the presence of glucose (0.6 mM) (filled bars) and mannose (0.6 mM) (striped bars) and in the absence of monosaccharides (open bars) The cells were incubated for 15 min 5 expts were performed each in duplicate samples Mean values + S E are shown

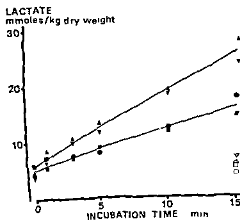


Fig 7 The time course for lactate accumulation in suspensions of isolated rat mast cells incubated with glucose (0.5 mM) in the presence (▲——▲ and ▼——▼ respectively) and absence (●——● and ■——■ respectively) of antimycin A (1.4×10^{-7} M) 2 experiments were performed each in duplicate samples The regression lines have been calculated according to the method of least squares Corresponding open symbols represent controls incubated for 15 min in the absence of glucose

Discussion

The present investigation shows that there is a difference in effect on rat mast cells between on the one hand D(+)glucose and D(+)mannose and on the other hand D(−)fructose, D(+)galactose, L-glucose, D(−)ribose and 2 deoxy D ribose Thus only glucose and mannose enhanced ATP-induced histamine release and only these hexoses were metabolized through the Embden Meyerhof pathway by the rat mast cells as judged from the lactate accumulation The effect of mannose was not due to a contamination with glucose

In most tissues mannose is thought to enter the Embden Meyerhof pathway because it is a good substrate for hexokinase Mannose 6-phosphate thus formed is then isomerized to fructose 6-phosphate by a specific mannose phosphateisomerase (Sols 1968)

The reports in the literature about the metabolism of fructose and galactose in mammalian tissues are somewhat conflicting There seems to be great variations between different tissues depending on the distribution of the various enzymes involved in the metabolism According to Chakravarty (1968) ^{14}C-fructose and ^{14}C-galactose as well as ^{14}C glucose are taken up by rat mast cells and metabolized by the oxidative pathway as judged from the production of ^{14}CO Glucose was oxidized about twice as fast as fructose The oxidation rate of galactose was quite high but could not be directly compared with that of glucose, because of differences in labelling of the substrates

Our results indicate that neither fructose nor galactose are metabolized through the Embden Meyerhof pathway The present results regarding the metabolism of hexoses by rat mast cells are in agreement with the findings of Diamant and Uvnäs (1961) that glucose, but not fructose or galactose restored compound 48/80-induced histamine release inhibited by anoxia

When comparing our results with those reported for insulin secretion Grodsky *et al* (1963) and Coore and Randle (1964) showed that insulin release from perfused rat pancreas and from incubations of pieces of rabbit pancreas was stimulated by the presence of glucose (above 3 mM) and mannose (above 6 mM) 18 mM of fructose did not stimulate insulin release when present in incubations of pieces of rabbit pancreas, but 27 mM of fructose had a slight stimulating effect on insulin secretion from perfused rat pancreas Galactose and ribose did not stimulate insulin secretion in either system

Jarret and Keen (1968) found that incubation of isolated rat pancreatic islets with labelled hexoses resulted in the production of ^{14}CO Glucose had a more pronounced effect than mannose which in turn was more effective than fructose On the other hand galactose did not give any production of ^{14}CO Hellerstrom (1967) found the same pattern with regard to the ability of the hexoses mentioned above to stimulate oxygen consumption of isolated pancreatic islets of mice

The finding that only those monosaccharides which induced lactate accumulation in suspensions of rat mast cells enhanced ATP induced histamine release raises the question if lactate *per se* is the cause of the enhancement Since we do not kno

the intracellular concentration of lactic acid in the mast cells neither after extracellular addition of lactate, nor after intracellular production we can not exclude that lactic acid by itself exerts a direct enhancing effect The same argument however, could be raised for any other metabolite of glucose that could be demonstrated to accumulate Certain experimental data, however, do not support the suggestion that the enhanced histamine release was due to lactic acid *per se* Firstly, an increased lactate accumulation was observed when the mast cells were incubated with glucose in the presence of antimycin A as compared to the values found in the absence of the inhibitor In spite of the increased lactate accumulation no histamine release was induced by ATP in the presence of antimycin A Secondly, assuming free diffusion of lactic acid across the cell membrane, the intracellular concentrations of lactic acid in the presence of glucose and mannose would not exceed 10^{-7} M in the experiments concerning histamine release and this concentration of lactate had no enhancing effect when applied extracellularly It is also evident that histamine release was potentiated by glucose in concentrations which did not give rise to lactate accumulation.

Chakravarty (1965) calculated the lactate production of isolated rat mast cells assuming that all CO_2 obtained anaerobically in a bicarbonate medium was produced by glycolytic lactic acid formation. In the absence of substrate a lactic acid production of 6.38×10^{-14} moles $\times$ cell^{-1} $\times$ h^{-1} was calculated In the presence of 5 mM of glucose the production increased slightly but statistically not significant to $7.60 \times 10^{-14} \times$ cell^{-1} $\times$ h^{-1} From our results a lactic acid production amounting to 0.5×10^{-14} moles $\times$ cell^{-1} $\times$ h^{-1} could be calculated from cells incubated in the presence of antimycin A but in the absence of metabolic substrates However the addition of 0.6 mM of glucose to such cells gave rise to a 15 fold increase of the production of lactic acid (7.6×10^{-14} moles $\times$ cell^{-1} $\times$ h^{-1}) Thus in contrast to Chakravarty we found that glucose induced a marked increase in the production of lactic acid from mast cells devoid of a functioning oxidative phosphorylation The reason for this difference in the two investigations is so far not clear However our results seem better in accordance with the view that an increased glycolytic energy production is the main reason why glucose restores 48/80 induced histamine release from mast cells inhibited by anoxia or inhibitors of oxidative phosphorylation (Diamant and Uvnäs 1961 Diamant and Krüger 1967)

Histamine release induced by ATP was inhibited by inhibition of oxidative phosphorylation (antimycin A as well as by inhibition of glycolysis (iodoacetate) and glucose failed to counteract either of the inhibitors However the inhibition exerted by iodoacetate was counteracted by the addition of metabolites of glucose entering the metabolic pathway below the site of the inhibition These findings strongly point to the *importance* of a functioning oxidative phosphorylation in the mast cells for histamine release to be induced by extracellularly applied ATP Since compound 48/80-induced histamine release inhibited by inhibitors of oxidative phosphorylation was promptly counteracted by glucose (Diamant and Krüger 1967) there exist a distinct difference between the two histamine releasing agents in that glycolytic

reactions seem to suffice to deliver energy to the histamine releasing process caused by 48/80, but not by ATP

Lactate was not found to accumulate in suspensions of mast cells during incubation at +37° C for 15 min in the absence of metabolic substrates and inhibitors In contrast when mast cells were incubated in the presence of an inhibitor of oxidative phosphorylation a slight, but statistically significant accumulation of lactate was observed This indicates that the energy supply of isolated rat mast cells at least partially is maintained by active glycolysis using endogenous substrates This is further supported by the finding that the endogenous ATP-content of isolated rat mast cells remained unaffected after incubation at +37° C for 60 min in the absence of added metabolic substrates (Diamant 1967)

The finding that histamine release was enhanced at concentrations of glucose which did not induce lactate accumulation indicates that the rate of pyruvate oxidation was not limited In that way increasing the oxidation of pyruvate should increase the oxidative generation of ATP, accounting for the enhancement by glucose of histamine release induced by exogenously applied ATP When the glucose concentration was increased above 0 06 mM, the capacity of the mast cells to oxidize pyruvate seemed to be exceeded since lactate started to accumulate in increasing amounts depending on the glucose concentration up to 0 4 mM Further increase in glucose concentration was not accompanied by any additional lactate accumulation which could indicate either that the maximal velocity of the lactic dehydrogenase reaction in the cells had been reached or that some other glycolytic reaction had become rate limiting or that the mechanism of glucose transport into the cells had been saturated

The results support the suggestion that an increase of the aerobic metabolic rate enhances histamine release from isolated mast cells induced by exogenously applied ATP (Diamant and Peterson 1970)

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Effects of Noradrenaline on Activity in Single Aortic Baroreceptor Fibres

By

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Abstract

AARS, H *Effects of noradrenaline on activity in single aortic baroreceptor fibres* Acta physiol scand 1971 *83* 335—343

The possibility of sympathetic control of the sensitivity of arterial baroreceptors has been examined by recording activity of single baroreceptor fibres in the rabbit's aortic nerve before and during intravenous infusion of noradrenaline (4—6 μg/kg min) Activity was related to blood pressure — which could be changed by bleeding and reinfusion of blood — and to diameter of ascending aorta measured with ultrasonic technique inside the intact thorax At the same blood pressures as in control runs, activity of 34 single myelinated fibres (conduction velocities 10—35 m/s) was lowered by noradrenaline, on average by 1 spike per pulse beat and diastolic aort[illegible] tolic diamete[illegible] ins but eve[illegible] to 50 % [illegible] ved higher than normal activity also at comparable blood pressures The results demonstrate that the sensitivity of aortic baroreceptors to distension of the receptor area can be increased by noradrenaline However, as aortic diameter is simultaneously decreased, activity in most single baroreceptor fibres was slightly reduced at any given blood pressure during intravenous infusion of noradrenaline

Local application of catecholamines to a baroreceptor area is known to produce reflex reduction of arterial blood pressures (Heymans and Neil 1958) To many investigators this response has suggested the existence of a sympathetic control of the sensitivity of arterial baroreceptors The hypothesis has mainly been tested for the carotid sinus receptors by recording effects of catecholamines or stimulation of sympathetic fibres to the sinus on activity in single or multiple baroreceptor fibres (Landgren, Neil and Zotterman 1951 Floyd and Neil 1952 Landgren 1952 Peterson 1966 Koizumi and Sato 1969, Sampson and Mills 1970) Results are conflicting possibly because dissection of the area had interfered with the smooth muscle tone of the carotid sinus

For the baroreceptors of the aortic arch investigations in the rabbit showed that the relationship between blood pressure and activity of the left aortic nerve was unchanged by infusion of noradrenaline as aortic diameter was simultaneously re-

duced, the results suggested that noradrenaline had increased the sensitivity of these receptors to stretch (Aars 1971 a) In that study, baroreceptor activity was measured as integrated activity of the whole left aortic nerve — a technique known to favour activity in the thickest myelinated fibres Baroceptor nerves, however, contain myelinated fibres of varied thickness as well as nonmyelinated fibres The influence of intravenous infusions of noradrenaline has therefore been examined by recording activity in single fibres of the aortic nerve Receptor activity was related to arterial blood pressure and aortic diameter, measured close to the receptor area within the intact thorax

Material and methods

Adult rabbits were anesthetized with a mixture of chloralose and urethane (Aars 1971 b) A heating lamp and covers were used to keep rectal temperature between 36° and 38° C The animals were tracheotomized but respiration was unassisted

Single nerve fibres were dissected from the caudal part of the cut left aortic nerve in the neck and action potentials were recorded with two platinum electrodes and a differential amplifier (Tektronix 122) The signals were displayed on an oscilloscope and recorded on a jet ink writer (Elema Mingograph) Conduction velocity was measured in most fibres by stimulating the nerve about 20 mm distal to the recording site

Diameter of the ascending aorta was measured as the transit time of ultrasonic pulses between two crystals stitched to the aortic wall (Aars 1969 1971 b) The crystals had been implanted 3—15 days earlier (average 10 days) under pentobarbital and ether anesthesia. Recordings of aortic diameter were made on a Sanborn recorder The diameter varied with the size of the animal In order to compare the receptor activity to aortic diameter the diameter was expressed in per cent of diastolic diameter at resting control diastolic blood pressure (which ranged from 68 to 95 mm Hg)

Blood pressure was measured in the right common carotid artery through a catheter connected to a Statham transducer (P23Gb) and recorded on both recorders Paper speed was 100 mm/s

During the experiment arterial blood pressure was changed by stepwise hemorrhage and retransfusion either through the right carotid artery or through a reservoir connected to the abdominal aorta Recordings were started after about 30 s at each new pressure level when a steady state had been reached Activity in single baroreceptor fibres was then counted on the neurogram for 10 consecutive pulse beats blood pressure and diameter for two and the mean figures calculated Immediately after [illegible] ed at various blood pressure levels during infusion of 4— [illegible] vein Recording of activity began about [illegible] Aortic diameter and receptor activity usu [illegible] during hemorrhage as during retransfusion When differences did occur observations with noradrenaline were compared to control observations obtained during the same direction of change When several preparations were tested in the same animal at least 30 min elapsed between each test to ensure that the effect of the former noradrenaline infusion had passed Most preparations were tested for chemoreceptor fibres by subjecting the animal to respiration of 20 % CO_2 in air

Results

Successful recordings of single fibre activity aortic blood pressure and aortic diameter, before and during infusion of noradrenaline were made in 15 rabbits Results are presented for three different groups of myelinated fibres (1) 34 thick and medium sized A fibres (conduction velocities 10—35 m/s) with high amplitude action potentials and low pressure receptor thresholds at about 30—50 mm Hg diastolic pressure, (2) 5 A fibres with similar conduction velocities and spike ampli

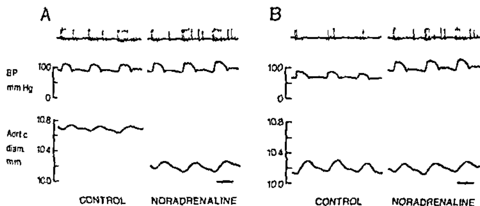


Fig 1 Baroreceptor activity in the left aortic nerve (single fibre preparation) in relation to arterial blood pressu
6 µg/kg min norad
at equal diastolic blo
diameter altered by
activity is unchange
pressure increased when compared at same aortic diameter

RATE OF CHANGE OF DIAMETER dD/dt (mm/sec)

PULSATILE DIAMETER VARIATIONS ΔD (mm)

SINGLE FIBRE ACTIVITY (spikes/pulse beat)

control

noradrenaline

DIASTOLIC BLOOD PRESSURE (mm Hg)

DIASTOLIC AORTIC DIAMETER (mm)

Fig 2 Effect of noradrenaline 6 µg/kg min) on activity of single aortic baroreceptor fibre Solid symbols Control observations during stepwise bleeding and reinfusion of blood Open symbols During infusion of noradrenaline and alterations in blood volume

Fig 3 Single fibre activity amplitude (ΔD) and maximum rate of change (dD/dt) of pulsatile diameter variations at various diastolic aortic diameters in the control state (solid symbols) and during infusion of 6 µg/kg min noradrenaline (open symbols Same fibre as shown in Fig 2

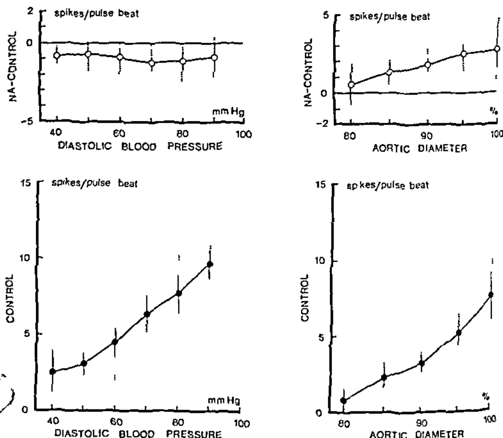


Fig 4 Effect of noradrenaline (4—6 μg/kg min) on activity in 34 single baroreceptor fibres of left aortic nerve in relation to blood pressure *Upper part* Difference between activity with noradrenaline (NA) and in the control state *Lower part* Control activity Vertical full lines denote ± 2 SE dotted lines ± 1 SD

tudes as in the first group but apparently without low pressure thresholds, (3) 13 nerve preparations which in addition to spikes from one or two thick A fibres contained low-amplitude spikes that could not be quantitated Conduction velocities of these fibres were about 5 m/s Unmyelinated C-fibres, with conduction velocities 2 m/s or less were repeatedly observed during dissection of the nerve *slips* but unfortunately these fibres always disappeared before the slip had been reduced to contain 1—3 active fibres Only one chemoreceptor fibre was found

In the main group of A fibres the number of spikes per heart beat at a given diastolic blood pressure was usually unchanged or slightly reduced by noradrenaline This can be seen from Fig 1 A in which action potentials from a single A fibre and diameter of ascending aorta have been recorded at roughly equal blood pressures

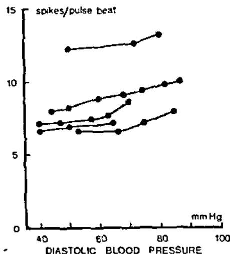


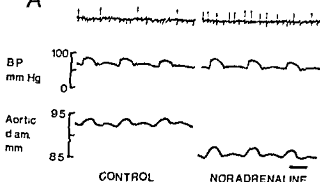
Fig 6 Activity of 5 aortic baroreceptor fibres plotted against blood pressure and related to range of activity (shaded area) of the population shown in Fig 4, lower part

before (control) and during infusion of noradrenaline At this pressure level, noradrenaline had reduced aortic diameter by about 5 % When compared to control observations at the same aortic diameter, but lower blood pressure (Fig 1 B), activity in the single fibre was found to be increased by noradrenaline

Activity of another similar A-fibre plotted against blood pressure is shown in Fig 2 Again, activity was unchanged or reduced by noradrenaline at equal blood pressures At equal diastolic aortic diameters (Fig 3), activity was higher than in the control run The rate of firing of baroreceptors is known to be influenced not only by diastolic diameter but by the amplitude of the pulsatile diameter variations (ΔD) and the rate of change of the diameter (dD/dt) These parameters are included in Fig 3, which shows that noradrenaline increased single fibre activity at equal diastolic diameters despite smaller ΔD and maximum dD/dt than in the control run

Fig 4 presents average baroreceptor activity in the main group of single A-fibres The receptors showed very uniform sensitivity to pressure, and the deviation around mean activity in control runs was mainly caused by differences in threshold (Fig 4 lower part) At all pressures, noradrenaline usually caused a slight reduction in the number of action potentials per pulse beat (Fig 4 upper part) Mean difference from control was significant at pressures from 40—80 mm Hg ($P < 0.02$) but small (about one spike per pulse beat) compared with the standard deviation of control activity at the same blood pressure Noradrenaline caused no consistent changes of mean heart rate, but at all pressures reduced diastolic aortic diameter by an average of 6 8 %

In Fig 5, activity of the same fibres has been compared at equal diastolic aortic diameters In the majority of observations from all fibres activity was increased by noradrenaline The mean difference between activity with noradrenaline and in the control run increased with diameter (Fig 5 upper part and was statistically si



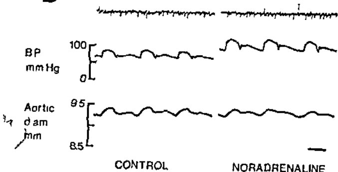


Fig 7 Baroreceptor activity in left aortic nerve (single fibre preparation), arterial blood pressure and diameter of ascending aorta, before and during infusion of 5 μg/kg min noradrenaline Conduction velocity of this fibre was about 30 m/s *7A* Compared at equal diastolic blood pressure. *7B* Compared at equal diastolic aortic diameter Pressure and diameter altered by adjustments of blood volume Horizontal bar = 1/10 s Note the more uniform distribution of action potentials than for the fibre shown in Fig 1 and that receptor activity is slightly reduced by noradrenaline when compared to control observations at same blood pressure (A) Activity is increased when compared at same aortic diameter (B)

cant ($P < 0\ 001$) above an aortic diameter of 80 % The difference approximately equalled one standard deviation of control observations (Fig 5 lower part) At 95 % and 100 % diameter, the increase amounted to 2 6 and 2 8 spikes per beat, i e, about 50 % and 37 % respectively Activity during a run with noradrenaline corresponded to activity at a 5 % larger diastolic aortic diameter during the control run Pulsatile variations of aortic diameter (ΔD) were unchanged at equal diastolic diameters in 34 % of observations during infusion of noradrenaline and reduced in 61 %, but no alteration occurred in the relationship between ΔD and maximum rate of change (dD/dt)

Activity of the 5 large-amplitude A-fibres of the second group showed minimal reductions when pressure was lowered to 40—50 mm Hg These receptors thus had a reduced sensitivity to pressure and a lower, if any, low-pressure threshold than the majority of receptors This is illustrated in Fig 6, in which control observations from these five fibres and the range of activity in the larger population (shaded area) are plotted against blood pressure As shown for a typical fibre in Fig 7, activity in these fibres was also more uniformly distributed throughout the pulse cycle, particularly at low pressures However, a pulsatile pattern of activity dominated at pressures above resting control levels and the fibres were classified as transmitting baro-

receptor impulses Their response to noradrenaline in no way differed from that of the larger population, either when activity was compared at equal blood pressure or at equal diameter (Fig 7A B)

The response to noradrenaline of receptors with the low amplitude spikes could not be quantitated The recordings did, however, allow a decision to be made as to whether activity in these fibres had been changed by noradrenaline In 6 of these preparations activity at a given blood pressure was largely unaltered by noradrenaline In 7 other cases, noradrenaline markedly increased activity in the low-amplitude fibres at equal blood pressures

Discussion

At any arterial pressure, the number of spikes per pulse beat in 39 medium sized and thick baroreceptor fibres was usually slightly reduced by infusion of noradrenaline The effect is the same if activity is judged as impulses per second because noradrenaline had no consistent effect on heart rate At equal diastolic aortic diameters noradrenaline produced a rise of activity However, as pulsatile variations of aortic diameter were frequently reduced, the results imply that with equal pulsatile variations receptor activity would have been even more increased In reality, noradrenaline therefore caused a larger increase in receptor sensitivity to stretch than shown in Fig 5

The increase in receptor sensitivity was higher than that found in the whole aortic nerve (Aars 1971 a) and activity at a given blood pressure — which in the former study remained unaltered during noradrenaline infusion — was reduced The discrepancy most likely reflected the use of more uniform receptor populations in the present study, and possibly also the higher infusion rates of noradrenaline (4—6 compared to 1—6 μg/kg min in the earlier study)

In contrast to the present studies of aortic receptors Peterson (1966) in a preparation of the sinus nerve, found that application of noradrenaline to the sinus caused a parallel reduction of vessel wall strain and repetition rate of large baroreceptor spikes Receptor sensitivity to stretch was thus unaffected by noradrenaline Conversely Landgren *et al* (1951) observed a small increase in impulse frequency of large baroreceptor spikes in response to local application of adrenaline Landgren (1952) subsequently showed that the frequency of one large baroreceptor spike was slightly reduced by adrenaline at steady pressures below 90 mm Hg *i e* pressures at which distensibility of the sinus ($\Delta D/\Delta P$) was reduced At higher pressures adrenaline increased distensibility and activity The activity was not related to actual diameter of the sinus Differences in response to catecholamines in the carotid sinus and the aortic arch might be explained by structural variations in the vessel walls other types of receptors or interference with smooth muscle tone due to dissection of the carotid sinus area The latter might also have contributed to the discrepancies between investigations of the carotid sinus baroreceptors

In both aortic and sinus nerves the frequency of some low amplitude baroreceptor-

spikes appears to be increased by noradrenaline (Landgren *et al* 1951, Landgren 1952 Peterson 1966) It is important to realize, however, that for the aortic nerve these low amplitude spikes belong to the group of slow conducting A fibres (for fibre composition of the aortic nerve, see O Leary, Heinbecker and Bishop 1934 Agostoni *et al* 1957 Chiarini 1960, Paintal 1963) Conduction velocity of the fibres was not determined by Landgren (1951, 1952) or Peterson (1966)

For the carotid sinus baroreceptors, a short (16—18 ms) latency of response to electrical stimulation of sympathetic fibres in the opposum suggested a direct effect on the receptors (Koizumi and Sato 1969) Sampson and Mills (1970) found that sympathetic stimulation could increase impulse frequency of these receptors also in cats (in 50 % of the fibres examined), but that the effect took several seconds to develop Direct action on aortic receptor endings by noradrenaline appears improbable, because of the tendency to reduce activity in thick myelinated fibres and increase activity in the thinnest myelinated fibres at any given blood pressure It would be reasonable to suppose that the dissociation indicates different coupling of the receptors in the aortic wall, and that the effect of noradrenaline is mediated by contraction of the smooth muscle tissue Receptors with thin fibres would accordingly be coupled in sereis with smooth muscle fibres as originally suggested for the carotid sinus by Landgren (1952) In contrast to other investigations (Landgren 1952 Peterson 1966), the results of the present study imply that receptors with thick A fibres are also to a certain extent influenced by smooth muscle tone

Available literature makes no reference to aortic baroreceptors with high activity even at low blood pressures These receptors differed from others also with regard to sensitivity to pressure and cyclic distribution of action potentials Maintained activity in these fibres might be one reason why activity in the whole left aortic nerve does not disappear completely at low pressures (Aars 1968) These fibres may also be responsible for the marked increase of efferent sympathetic nerve activity which follows complete barodenervation at low arterial blood pressures in the rabbit (Aars and Akre 1971) In that event the strong reflex effect and relatively small numbers of these baroreceptor fibres would indicate that they convey very potent sympathetic inhibitory impulses

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The Effect of Acute and Chronic Hypercapnia upon the Lactate, Pyruvate, α-ketoglutarate, Glutamate and Phosphocreatine Contents of the Rat Brain

By

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Abstract

MESSETER K and B K SIESJO *The effect of acute and chronic hypercapnia upon the lactate, pyruvate, a ketoglutarate, glutamate and phosphocreatine contents of the rat brain* Acta physiol scand 1971 83 344—351

The influence of hypercapnia upon the tissue contents of lactate pyruvate α ketoglutarate glutamate and phosphocreatine was studied in rats exposed to about 11 % CO_2 for 15 and 45 min and for 3 24 48 and 72 h respectively Acute hypercapnia (15 and 45 min) was associated with highly significant decreases in the lactate pyruvate α ketoglutarate glutamate and phosphocreatine contents In sustained hypercapnia (3 h and onwards) the lactate pyruvate and α ketoglutarate contents were partially restored but phosphocreatine and glutamate remained decreased In acute hypercapnia the intracellular lactate/pyruvate ratio was increased but it returned to normal in sustained hypercapnia (> 24 h) The results suggest that the intracellular lactate/pyruvate ratio is affected both by changes in the intracellular pH and by changes in the NADH/NAD ratio

Recent results from the laboratory have demonstrated that when rats were exposed to sustained hypercapnia (about 11 % CO_2) there was a gradual increase in the intracellular bicarbonate concentration of sufficient magnitude to bring the intracellular pH (pH) back close to the normocapnic control value (Messeter and Siesjo 1970, 1971 a) The regulation of pH′ occurred mainly during the first three hours and prolongation of the hypercapnia to 72 h did not lead to further acid base changes Since the pH regulation was far in excess of what could be due to physicochemical buffering it appeared probable that metabolic consumption of acids and/or transmembrane fluxes of H or HCO_3 played a role (Siesjo and Messeter 1971)

The present work which deals with the influence of the hypercapnia upon simultaneously measured cerebral metabolites had two objectives The first objective was to study if the hypercapnia gave rise to metabolic changes which by themselves could serve as pH regulating mechanisms Thus the effect of acidosis on the phosphofructokinase reaction (Delcher and Shipp 1966) suggests that a depletion of substrates could occur beyond this step and if this involves complete oxidation of

carboxylic acids and of diprotic monoamino acids, an equivalent amount of H^+ ions should disappear from the system It has previously been demonstrated that the tissue concentrations of lactate and pyruvate decrease in acute hypercapnia (Leusen *et al* 1967, Granholm and Siesjö 1969, Kjällquist *et al* 1969) In the present experiments the tissue concentrations of lactate and pyruvate, of a Krebs cycle acid (α keto glutarate) and of a metabolically active diprotic amino acid (glutamic acid) were measured in brains of animals exposed to carbon dioxide for periods of 15 min to 72 h and the changes observed could be related to the pH_i' values reported previously (Messeter and Siesjö 1971 a)

The second objective of the present work was to study if the changes in the intracellular pH, calculated from the CO_2 data, agreed with those predicted from two of the pH dependent equilibrium reactions of the tissue, *viz* the lactate dehydrogenase and the creatine phosphokinase reactions (Williamson *et al* 1967, Kuby and Noltman 1962) If the values would agree, these reactions may evidently be used to detect changes in pH_i' (*cf* Rose 1968) However, even if agreement is not obtained it should be important to establish the relation between, on one hand, the pH' and, on the other hand, the phosphocreatine concentration and the lactate/pyruvate ratio This is due to the fact that if these parameters should be considered to be valid indicators of the presence of tissue hypoxia it must first be established how they are influenced by tissue acidosis in the absence of hypoxia Previous results from the laboratory have shown that acute hypercapnia leads to a decreased phosphocreatine concentration (Granholm and Siesjö 1969, Siesjo and Messeter 1971) and to an increased lactate/pyruvate ratio (Granholm and Siesjö 1969) but the present experiments allowed correlation to the pH_i' changes occurring during continuous hypercapnia of maximally 72 hs' duration

Methods

The general procedures used for inducing the hypercapnia and the operative techniques were those described elsewhere (Messeter and Siesjo 1971 a and b) These publications give details of the measurements of the acid base parameters and of the calculation of intracellular pH The present measurements were obtained in the experiments covered by these communications Thus, in each individual brain analysed pH_i was known. However the size of the cerebro spinal fluid (CSF) samples precluded measurements of both bicarbonate and of lactate and pyruvate For that reason CSF lactate and pyruvate concentrations were measured in a small series of separate animals which were otherwise treated similarly

General outline of experiments

All experiments were performed on male rats of the Wistar strain weighing 300—450 g The animals were exposed to about 11 % CO_2 which was premixed with 30 % oxygen When hypercapnia was induced for 3 24 48 or 72 h respe
posed to the gas mixture in a perspex box and the
before the end of the exposure period In the group c
min the animals were anesthetized and maintained no
mm Hg) for 15—20 min before the gas mixture was delivered

Anesthesia was induced with divinyl ether and maintained with 0 6 % halothane after a quick tracheotomy and immobilization with tubocurarine chloride All animals were mechanically hyperventilated in order to bring the arterial CO_2 tension close to that observed in the unanesthetized animals breathing the CO_2-containing gas mixture (see Messeter and Siesjo 1971 b) The arterial blood pressure was measured in a cannula in one femoral artery and arterial samples were anerobically drawn from the cannula for measurements of the pH the

CO_2 tension and the O_2 tension The body temperature was kept close to 37° C by means of intermittent heating and the blood parameters measured were corrected for variations in body temperature from this value

cold instruments and stored at −80° C until analysed

Analytical techniques

For the measurements of the CSF, or the blood, lactate and pyruvate concentrations the samples were extracted with 6 % perchloric acid at 0—2° C and subsequently analysed with specific enzymatic techniques (see below) The supratentorial parts of the brains (400—600 mg) were chrushed in liquid nitrogen and extracted with 3 M perchloric acid at −10° to −15° C (Lowry *et al* 1964) The tissue contents of phosphocreatine lactate pyruvate α-ketoglutarate and glutamate were measured with enzymatic techniques using Zeiss PMQ II spectrophotometers and continuous recording of each enzymatic curve (for references and further details see Hindfelt and Siesjo 1971)

The concentrations of the metabolites were expressed in mmoles/kg of wet weight However when deriving the intracellular pH_i bicarbonate was calculated per kg of i c water using corrections for the bicarbonate contained in a 3 % blood and a 15 % extracellular space (ECF) (see Siesjo and Messeter 1971 Messeter and Siesjo 1971 a) Similar corrections were

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1—2 % should affect the calculations by a degree too small to affect any of the conclusions drawn corrections were omitted The intracellular pH_i and the intracellular lactate and pyruvate concentrations were used to calculate a cytoplasmatic NADH/NAD ratio according to the equation

$$\frac{NADH}{NAD^+} = \frac{Lact}{Pyr} \cdot \frac{K}{H^+}$$

where K was given a value of 1 11 · 10^{-11} (Williamson *et al* 1967)

Results

In all the present animals the mean arterial blood pressure exceeded 100 mm Hg and the arterial P_{O_2} varied between 100 and 150 mm Hg Since the influence of the hypercapnia upon the tissue acid base parameters is described in detail elsewhere (Messeter and Siesjo 1971 a) only the resulting pH′ values are reported below As described in that communication the administration of about 11 % CO_2 to the rats lead to an increase in the mean tissue CO_2 tension from 40—45 mm Hg to 85—90 mm Hg This degree of hypercapnia gave rise to an acute intracellular acidosis in the 15 min group with a decrease of pH′ from 7 06 to 6 93 When the hypercapnia was continued the pH_i′ was regulated back to 7 03 within 3 h with no further change during the 72 h observation period (see Messeter and Siesjo 1971 a)

Table I gives the mean values (±S E) for all the directly measured tissue parameters in the control group and in the hypercapnic groups together with the arterial CO_2 tensions and the intracellular pH′ values taken from the previous communication (Messeter and Siesjo 1971 a) The control values for lactate pyruvate and phosphocreatine agree closely with those published previously for the same anesthesia (Nilsson and Siesjo 1970) The α ketoglutarate values are very similar to

Exposure periods	P_{CO_2}	pH_i	La	Py	α KG	Glut	La/Py	PCr
Control (n = 9)	37.7 ±1.0	7.06 ±0.01	1.36 ±0.07	0.078 ±0.003	0.116 ±0.006	11.66 ±0.24	17.3 ±0.7	5.07 ±0.07
15 min (n = 10)	84.4 ±1.2	6.93 ±0.01	0.81 ±0.04	0.035 ±0.001	0.042 ±0.004	10.97 ±0.18	23.6 ±1.5	4.34 ±0.05
45 min (n = 9)	85.6 ±0.7	6.97 ±0.01	0.76 ±0.02	0.032 ±0.003	0.040 ±0.003	9.62 ±0.12	24.1 ±1.3	4.36 ±0.08
3 h (n = 9)	84.3 ±0.6	7.03 ±0.01	0.93 ±0.04	0.041 ±0.003	0.054 ±0.005	9.90 ±0.21	22.9 ±0.8	4.25 ±0.05
24 h (n = 7)	86.1 ±1.2	7.02 ±0.01	0.94 ±0.02	0.056 ±0.003	0.086 ±0.008	9.71 ±0.16	17.3 ±1.3	4.46 ±0.06
48 h (n = 8)	84.3 ±1.5	7.03 ±0.01	1.00 ±0.05	0.058 ±0.005	0.085 ±0.003	9.84 ±0.16	17.9 ±0.8	4.58 ±0.08
72 h (n = 8)	85.5 ±1.4	7.02 ±0.01	1.20 ±0.08	0.063 ±0.004	0.101 ±0.009	9.79 ±0.33	19.0 ±0.5	4.31 ±0.05

those obtained by Gatfield *et al* (1966 see also Hindfelt and Siesjo 1971) and the glutamate values agree with those of Folbergrova *et al* (1969) and of Hindfelt and Siesjo (1971)

Lactate pyruvate, α ketoglutarate and glutamate

Acute hypercapnia (15 and 45 min) gave rise to pronounced decreases in the tissue contents of these acids (Table I) The lactate content decreased maximally to 60 % of the control value, and the pyruvate and α ketoglutarate contents were reduced to less than half the normal values However whereas the contents of these acids did not decrease further after 15 min there was a further fall of glutamate in the 45 min group The behaviour of this acid differed from those of the others also in sustained hypercapnia Thus there was no further change in the glutamate content throughout the experimental period but a gradual return of the tissue contents of the other acids towards the control values

Intracellular lactate and pyruvate

In order to allow calculation of intracellular lactate and pyruvate concentrations the whole blood and the CSF lactate and pyruvate concentrations were measured in separate experiments (Table II) Although the number of measurements were small the results demonstrate that acute hypercapnia was associated with decreases in the blood and CSF lactate and pyruvate concentration and that the concentrations rose again in sustained hypercapnia In contrast to brain tissue however neither blood nor CSF showed an increase in the la[illegible]

TABLE II The measured lactate and pyruvate contents of cisternal cerebrospinal fluid of and of arterial blood (mmol/kg) together with the corresponding lactate/pyruvate ratios in similar groups (mmol/l) rats as shown in Table I (Means ± S E) There were 3–5 measurements in each group

CSF				Arterial blood		
Exposure periods to 11 % CO_2	Lactate	Pyruvate	La/Ly	Lactate	Pyruvate	La/Py
Control	2 98 ±0 13	0 192 ±0 008	15 8 ±1 4	1 47 ±0 15	0 122 ±0 013	12 1 ±0 4
15 min	1 90 ±0 13	0 113 ±0 009	17 0 ±0 9	0 75 ±0 15	0 074 ±0 012	10 0 ±1 0
45 min	1 70 ±0 1	0 102 ±0 005	16 9 ±1.2	0 98 ±0 16	0 088 ±0 009	10 8 ±1 0
3 h	1 72 ±0 06	0 104 ±0 005	16 6 ±0 4	0 88 ±0 0	0 076 ±0 0	11 7 ±0 4
24 + 48 h	2 13 ±0 14	0 107 ±0 005	19 8 ±0 6	1 59 ±0 32	0 127 ±0 022	12 3 ±1 1
72 h	2 57 ±0 23	0 118 ±0 004	21 7 ±1 7	2 81 ±0 49	0 178 ±0 028	16 0 ±1 9

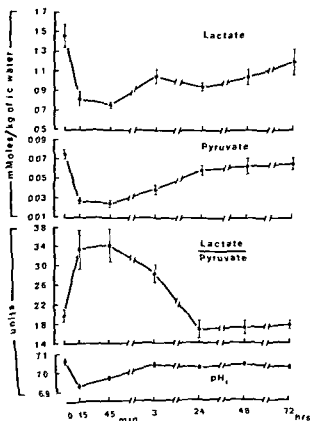


Fig 1 Time course of changes in the calculated intracellular lactate and pyruvate concentrations (mmol/kg of i c water) and lactate/pyruvate ratios compared to the corresponding calculated intracellular pH values in rats exposed to about 11 % CO_2 (means ±S E) Although the observed changes in acute hypercapnia (15 min and 45 min) may be explained by a pH effect on the rate of glycolysis and on the LDH equilibrium there were no obvious correlations with the intracellular acid base state after 45 min of hypercapnia

that under the experimental conditions the CSF lactate/pyruvate changes followed those of the blood, and not of the intracellular space

Fig 1 relates the calculated intracellular lactate and pyruvate concentrations, and the lactate/pyruvate ratios to the intracellular pH′ Although the lactate and pyruvate concentrations decreased acutely with pH′ there was no simple relationship between the changes in the concentrations of the acids and the changes in pH′ Thus although the calculated pH_i' did not change further after 3 h there was a further increase in pyruvate after that time

As predicted (see Introduction) the lactate/pyruvate ratio increased acutely when pH′ fell and there was a decrease to low values at 24 h and onwards However, there did not seem to be agreement between the fall in pH_i' and the increase in the lactate/pyruvate ratio since the 3 h group had a high lactate/pyruvate ratio with a relatively high pH_i' In order to illustrate this relationship further the pH_i' values were used to calculate expected changes in lactate/pyruvate ratio, assuming constant NADH/NAD ratio As shown by Fig 2 the actual lactate/pyruvate ratios increased above the expected ones during the first 3 h and decreased below the expected values at 24 h and onwards The differences calculated probably indicate that the cyto plasmatic NADH/NAD ratio varies in hypercapnia (see Discussion)

The phosphocreatine content Provided we may define an equilibrium reaction for the creatine phosphokinase reaction based on the total tissue contents of ATP, ADP, phosphocreatine and creatine (Cr) the apparent equilibrium constant is given by the equation (Kuby and Noltman 1962)

$$K' = \frac{(ATP)}{(ADP)} \frac{(Cr)}{(PCr)} \frac{1}{(H^+)} \quad (2)$$

In the present control groups our (unpublished) ATP and ADP concentrations were 2 82 and 0 37 mmol/kg respectively and other experiments showed a creatine concentration of 5 5 mmol/kg (*cf* Granholm *et al* 1968) With these concen trations K can be calculated to 9 48 10^7 l mol^{-1} Since the ATP/ADP ratios were found to remain unaltered during the first 3 h (unpublished results), we can deduce

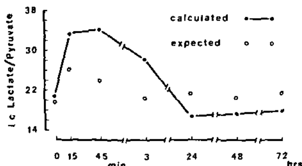


Fig 2 A comparison between the observed and the expected cytoplasmatic lactate/pyruvate ratios in acute and sustained hypercapnia The expected cytoplasmatic lactate/pyruvate ratios (unfilled circles) were calculated on the tentative assumption that the NADH/NAD ratio remained unchanged in acute and sustained hypercapnia The figure suggests that the changes in the observed lactate/pyruvate ratios (filled circles) could not be satisfactorily accounted for by the changes in the apparent intracellular pH during acute hypercapnia

from equation (2) that the phosphocreatine concentrations ought to be 4 27, 4.51 and 4 86 mmol/kg at 15 and 45 min and at 3 h respectively Table I shows that there was good agreement between the measured and predicted values at 15 min less so at 45 min, and a pronounced difference at 3 h The results demonstrate that the relationship between pH_i' and the phosphocreatine concentration is more complex than the above analysis would predict (see Discussion)

Discussion

The present results lend support to the assumption that the regulation of pH_i' in *acute hypercapnia may be partly due to removal of H when the pools of organic* acids are decreased as a result of the hypercapnia (Siesjö and Messeter 1971) Since the tissue concentrations of lactate, pyruvate, α ketoglutarate and glutamate decreased it is conceivable that also other acids were depleted However since the tissue concentrations of the first three of these acids returned towards normal in sustained hypercapnia other mechanisms, such as transmembrane fluxes of H or HCO_3 may be responsible for the accumulation of bicarbonate in the sustained state Although the results obtained in acute hypercapnia fit with the simple assumption that the acidosis causes a depletion of metabolites beyond a rate limiting step such as the phosphofructokinase reaction the results obtained in sustained hypercapnia indicate a more complex influence Thus neither the slow normalization of the lactate pyruvate and α ketoglutarate concentrations at a stable pH′ nor the difference in behaviour between these acids and glutamate can be explained that simply It will remain for further studies to explore other biochemical alterations induced by hypercapnia and the effects they may have on the regulation of pH′

The results obtained confirm the increases of the tissue lactate pyruvate ratio previously observed in acute hypercapnia (Granholm and Siesjo 1969 Kjallquist *et al* 1969 and they demonstrated a decrease of the ratio to normal or subnormal values in sustained hypercapnia Although the variations superficially agree with the changes in pH′ the increase in the lactate/pyruvate ratio was larger in acute hypercapnia and the secondary fall of the ratio in sustained hypercapnia was greater than what could be predicted from the calculated pH′ see also difference at 3 h in Fig 1) Since the lactate pyruvate ratios derived were either lager acute hypercapnia) or smaller sustained hypercapnia than those expected from the pH dependence of the LDH equilibrium the results do not indicate that the differences were due to an inaccurate equilibrium constant but they may be due to the fact that the cytoplasmatic NADH NAD ratios increased above the control during the first 3 h while a decrease to subnormal values occurred at 24 and 48 h Thus the intracellular lactate pyruvate ratio probably varied both with pH′ and with the NADH/NAD ratio

Our results confirm the decrease in the tissue phosphocreatine content previously observed in acute hypercapnia Granholm and Siesjo 1969 Siesjo and Messeter 1971) but they showed that the partial normalization of pH′ in sustained hypercapnia was unaccompanied by a corresponding normalization of phosphocreatine

The discrepancy could either be due to the fact that the sum of the phosphocreatine and creatine concentrations changed in sustained hypercapnia, or that the equilibrium constant varied due to *e g* changes in the free magnesium concentration (Kuby and Noltman 1962 Rose 1968) At any rate, the results demonstrate that acidosis leads to decreases in the phosphocreatine concentration in the absence of overt hypoxia, and they indicate that moderate decrease in the phosphocreatine concentration during hypoxia should be interpreted with caution

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Differential Effect of Sleep Deprivation on the Two Slow Wave Sleep Stages in the Cat

By

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Abstract

URSIN, R *Differential effect of sleep deprivation on the two slow wave sleep stages in the cat* Acta physiol scand 1971 *83* 352—361

[illegible] interrelations was studied in [illegible] h after 12 h and 24 h sleep [illegible] nto three stages *Light slow* [illegible] eye movement (REM) sleep [illegible] dings increased with deprivation as did the relative proportion (per cent of total sleep) of these sleep stages The total quantity of LSWS did not change with sleep deprivation and the LSWS per cent of total sleep decreased The changes were most pronounced after 24 h deprivation and in the first hours of [illegible] DSWS [illegible]

The sleep following a period of total sleep deprivation differs quantitatively from ordinary sleep Sleep deprivation is therefore a useful tool in studying the importance and possible relationship of the various sleep stages identified in a given species

Most research on sleep in the cat has regarded slow wave sleep (SWS) as an entity In humans it has appeared useful to identify several stages of slow wave sleep (Kales *et al* 1970) In a previous work (Ursin 1968) it has been shown that also in the cat such a differentiation is possible and functionally meaningful Two stages of slow wave sleep have been identified light slow wave sleep (LSWS) and deep slow wave sleep (DSWS) There is a specific relation between DSWS and rapid eye movement (REM) sleep while no such relationship exists for LSWS A '*trigger* control mechanism' was postulated from the finding that REM sleep almost always is preceded by DSWS A 'duration control mechanism" was suggested from the finding of a positive correlation between the inter REM content of DSWS and the length of the *preceding REM sleep period* (Ursin 1970) Evidence for this mechanism also derives from the positive correlation between total time spent in DSWS and REM sleep during a 24 h period (Ursin 1968)

The present report deals with the possible differential effect of total sleep deprivation on the two stages of slow wave sleep In man, both REM sleep and the deepest stage of slow wave sleep are changed in postdeprivation sleep (Berger and Oswald 1962 Williams *et al* 1964, Kales *et al* 1970) In the cat, the relative proportions of slow wave sleep and REM sleep are also changed after total sleep deprivation, although existing studies do not agree upon the direction of these changes and SWS has not been differentiated (Kiyono *et al* 1965, Vimont Vicary, Jouvet-Mounier et Delorme 1966) It seems reasonable to expect an increase of DSWS In accord with the earlier findings it is also postulated that an increase of DSWS will be accompanied by an increase of REM sleep and a decrease of LSWS

Materials and Methods

10 adult cats were used 5 were females The implantation procedure has been described in detail earlier (Ursin 1968) A11
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... was open eyes The deprivation was main ... mainly with light sensory stimulation (talking touching playing moving to another cage moving the cage) If this was not sufficient the cat was moved to a threadmill for up to 30 min The speed of the threadmill could be varied between 128 cm/min and 272 cm/min It was usually kept at low speed to avoid unnecessary muscular exercise This allowed the cat to walk quietly to the end of the band and ride down 75 cm before it had to move again to avoid a pool of water at the end of the band The
mill, and additional stimulation was giver
Sleep was recorded continuously for 24
sleep deprivation and with no precedin
between experiments ranged from 5 to 1. days and was usually 7 or 8 days The cats were habituated to the recording chamber by spending at least four ho
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ch) The cat was placed in the recording ... together with what was left of his daily ration of food and milk A new supply of food and milk was given when he was awake around 12 noon the following day This was the usual feeding time in the living quarters

Recordings were done with alternating speeds of 1 5 mm/s and 30 mm/s one page (30 cm) each Behavioral notes were taken each time the speed was changed to slow The criteria both for the
(Ursin 1968)
including drowsi
sleep spindles ar
slow wave sleep
spindles *REM s*
bursts of rapid e ... the 2 speeds a special set of criteria has been developed for reading the slow speed records Interobserver agreement is satisfactory with these sets of criteria (Ursin 1968)

All records were read by the same experimenter. The records were read in 1 min epochs by scoring clock time interval. When more than one stage was present in one epoch the stage that occupied most of the epoch was scored. Clock time and diagnosis were then punched on cards for computer processing.

Sandler's *A* statistics, which is based on the Student *t* ratio for correlated samples, was used in the statistical evaluation of sleep time changes (Runyon and Haber 1968). The FORTRAN subroutine SRANK was used in the sleep cycle correlation analysis. For the other correlated data the Pearson product moment correlation coefficient was determined (Runyon and Haber 1968).

Results

Total sleep time and composition of sleep

Total sleep time was relatively constant from cat to cat under the non deprivation condition (42.0% of recording time, S.D. = ±4.3). It was significantly increased both after 12 h and after 24 h deprivation ($P < 0.0005$). LSWS did not participate in this increase. Total quantity of this sleep stage was remarkably stable under the various conditions. DSWS and REM sleep quantities both increased with increasing sleep deprivation. The increases were significant ($P < 0.025$) both from no deprivation to 12 h deprivation and from 12 h to 24 h deprivation, and highly significant ($P < 0.0005$) from no deprivation to 24 h deprivation (Fig. 1). DSWS in the beginning of recovery sleep tended to contain slower waves of higher amplitude than regular DSWS.

Since total sleep time increased and LSWS did not participate in this increase there was a significant decrease of LSWS per cent of total sleep ($P < 0.005$) after 24 h deprivation. The total SWS per cent of total sleep decreased ($P < 0.005$) even though DSWS per cent increased ($P < 0.005$). Accordingly, REM sleep per cent was increased ($P < 0.005$).

There was a significant positive correlation ($P < 0.05$) between total quantity of DSWS and total quantity of REM sleep for all 3 conditions when comparing in

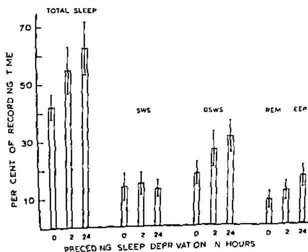


Fig. 1. Mean values (plus/minus one standard deviation) of total sleep, LSWS, DSWS and REM sleep within 24 h after no sleep deprivation, 12 h sleep deprivation and 24 h sleep deprivation.

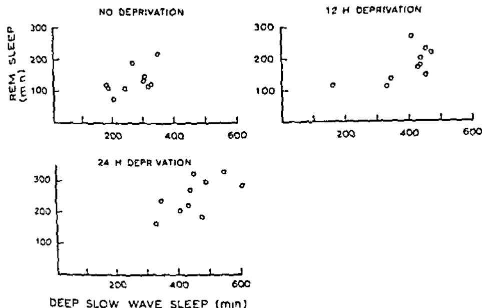


Fig 2 Relation between total quantities of DSWS and REM sleep in 24 h recordings after no deprivation 12 h and 24 h deprivation Each cat is represented by one circle in each of the 3 figures

dividual cats ($r = 0.58$, 0.60, 0.62 respectively Fig 2) The increases in DSWS and REM sleep from non deprivation sleep to post 24 h deprivation sleep were highly correlated The cats that showed a large DSWS increase also had a large increase of REM sleep ($r = 0.87$, $P < 0.0005$) The ratio REM sleep/DSWS was not significantly changed after sleep deprivation

Episode number and length

Each time a sleep stage started an episode of this sleep stage was counted The episode lasted until the sleep stage was interrupted by another sleep stage or by the awake state Waking episodes were counted in the same way The number of episodes increased with increasing sleep deprivation for all sleep stages (Table I) LSWS episodes were significantly shorter while DSWS episodes were significantly longer after 24 h deprivation REM sleep episodes tended to be longer after 24 h deprivation but this was not significant ($P < 0.10$)

Even though total sleep time increased there was no change in the number of waking episodes following sleep deprivation but the length of these episodes was significantly decreased both after 12 h and 24 h deprivation

First episodes Latencies and lengths

The time from start of recording until the first episodes of LSWS DSWS and REM sleep occurred *was shorter after* sleep deprivation than after no deprivation (Table II) However the quantity of SWS preceding the first REM sleep episode was not

TABLE I Number of episodes and episode length Means and significance level of differences (two tailed)

	0 h deprivation	12 h deprivation	24 h deprivation	0h—12 h P<	0h—24 h P<	12 h—24 h P<
Episode number						
Awake	81	87	79	Not sign	Not sign	Not sign
LSWS	109	129	124	0 001	0 05	Not sign
DSWS	76	101	104	0 001	0 001	Not sign
REM sleep	29	36	51	0 05	0 001	0 01
Episode length (min)						
Awake	10 98	7 65	7 07	0 001	0 001	Not sign
LSWS	1 82	1 71	1 56	0 05	0 01	0 02
DSWS	3 67	3 89	4 42	Not sign	0 05	Not sign
REM sleep	4 72	4 94	5 13	Not sign	Not sign	Not sign

TABLE II Latency to and length of first episode Mean values and significance level of differences (two tailed)

	0 h deprivation	12 h deprivation	24 h deprivation	0 h—12 h P<	0 h—24 h P<	12 h—24 h P<
Latency (min)						
LSWS	96	19	19	0 01	0 01	Not sign
DSWS	116	24	15	0 01	0 001	Not sign
REM sleep	210	103	49	0 02	0 01	0 02
Length (min)						
LSWS	3 0	1 5	1 9	Not sign	0 05	Not sign
DSWS	3 4	5 4	8 1	Not sign	0 01	Not sign
REM sleep	3 6	4 2	5 6	Not sign	Not sign	Not sign
Sleep before first REM (min)						
LSWS	26	15	6	Not sign	0 05	0 05
DSWS	15	29	22	0 05	Not sign	Not sign
Total SWS	41	44	28	Not sign	Not sign	Not sign

significantly changed with deprivation There were some changes in the composition of sleep towards less LSWS and more DSWS before the first REM sleep episode occurred after deprivation but only some values reached significance (Table II lower part) The significant decrease in latency to the first REM sleep episode, then seemed mainly to be due to the reduction of the time awake

Sleep stage alternation

Sleep stage alternation in the normal sleep of the cat has been described previously (Ursin 1970) The main line of stage alternation, from awake to LSWS to DSWS to REM sleep, was unchanged after sleep deprivation *Table III* gives the sequence

TABLE III Sleep stage alternation Mean for 10 cats

Preceding stage				Stage	Succeeding stage			
Awake %	LSWS %	DSWS %	REM S %		Awake %	LSWS %	DSWS %	REM S %
				No deprivation				
	42	29	29	Awake (N = 81)		91	8	0
67		27	5	LSWS (N = 109)	31		64	6
9	91		1	DSWS (N = 76)	31	39		30
0	21	79		REM S (N = 29)	78	20	2	
				24 h deprivation				
	28*	34	38*	Awake (N = 79)		86	13	0
54**		31	15**	LSWS (N = 124)	18*		73	9*
10	88		3	DSWS (N = 104)	26*	37		37*
0	22	77		REM S (N = 51)	58**	37*	5	

** $P<0.01$ two-tailed
* $P<0.05$ two-tailed

of sleep stages after no deprivation and after 24 h deprivation Most of the changes reflect the fact that there were more sleep episodes and relatively fewer awake episodes after 24 h sleep deprivation Accordingly, there was less fluctuation between the awake state and the sleep stages, the sleep episodes were more often succeeded by sleep and less often by awake episodes

The sleep cycles

A sleep cycle was defined as the time from the start of one REM sleep episode to the start of the next REM sleep episode Inter REM periods of 3 min or less were judged to be REM sleep interruptions' and excluded (Ursin 1970) Cycles that contained more than 10 min of the awake stage were also excluded in the calculation of sleep cycle length This was done to avoid distortion of sleep cycle length by long waking periods The cycle length did not change with deprivation (Table IV) Awake content in these cycles was decreasing with increasing deprivation The number of cycles was slightly increased after 12 h deprivation and significantly increased after 24 h deprivation

The number of excluded cycles did not vary with deprivational state but the amount of time awake in these cycles was significantly lower after both 12 h and 24 h sleep deprivation Again it is clear that the number of awake periods was not changed it was the duration of each episode that was shortened after sleep deprivation

Sleep stage variation throughout the 24 h recording period

The distribution of the sleep stages throughout the 24 h period of recording was studied by determining the quantity of each sleep stage per 3 h period (Fig 3) The increase in minutes of DSWS and REM sleep with 24 h deprivation as compare

TABLE IV Length and number of sleep cycles Mean values, standard deviations and significance levels of the differences (two tailed)

	0 h deprivation	12 h deprivation	24 h deprivation	0 h—12 h P<	0 h—24 h P<	12 h—24 h P<
Cycles with Awake < 10 min						
Length (min)	21 25 ± 3 70	21 34 ± 2 62	19 68 ± 1 76	Not sign	Not sign	Not sign
Number	15 4	20 9	34 3	Not sign	0 005	0 01
Awake (min)	3 81	3 09	2 37	0 05	0 01	0 01
Excluded cycles						
Awake > 10 min						
Length (min)	89 69 ± 22 04	77 71 ± 16 73	74 19 ± 17 59	Not sign	0 05	Not sign
Number	10 4	11 1	8 9	Not sign	Not sign	Not sign
Awake (min)	58 9	46 5	44 1	0 05	0 01	Not sign

with no deprivation sleep was most pronounced in the first three 3 h periods of recovery sleep ($P < 0.005$), but was significant also for period no 5 and 6 ($P < 0.025$) This increase in DSWS and REM sleep time was at the expence of waking time, LSWS time being unchanged

There was a slightly slower start of REM sleep rebound than of DSWS rebound within the first 3 h period The quantity of REM sleep in the very first hour of recovery sleep after 24 h deprivation was significantly lower than in the second hour (means were 8, 17 and 14 min in the first 3 h) DSWS quantities per hour in the first 3 h period did not vary significantly (means were 27, 28 and 27 min) Apart from this, the two stages seemed to follow each other throughout the recovery sleep Fig 3 shows that under the same conditions, DSWS and REM sleep generally peaked in the same 3 h periods The relationship between the two stages was evident not only in the 3 h periods but also when hourly quantities were compared There were significant positive correlations between the mean hourly quantities of DSWS and REM sleep under all three conditions (no deprivation $r = 0.72$, $P < 0.0005$, 12 h deprivation $r = 0.43$, $P < 0.025$, 24 h deprivation $r = 0.73$ $P < 0.0005$)

The results after 12 h deprivation were, with a few exceptions intermediate to the results after no deprivation and 24 h deprivation mentioned above (Fig 3)

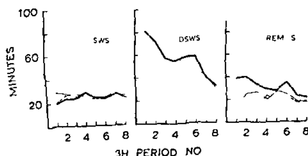


Fig 3 Minutes of LSWS DSWS and REM sleep per 3 h period of recording after no deprivation (broken line), 12 h deprivation (thin line) and 24 h deprivation (thick line)

Intracycle correlations

In a previous report (Ursin 1968), analysing 48 h recordings it was demonstrated that the length of a REM sleep episode was positively correlated to the quantity of DSWS in the succeeding inter-REM period Sleep deprivation did not seem to affect these intracycle mechanisms In the present 24 h recordings, the correlation between length of a REM sleep episode and quantity of succeeding DSWS was positive for all 3 experimental sessions in nine cats One cat had a negative correlation after 12 h deprivation positive after 0 h and 24 h deprivation The correlation coefficients did not differ from those of the previous material but only some of them reached significance Two after no deprivation, four after 12 h deprivation and five after 24 h deprivation This apparent increase in significant correlations with increasing deprivation is probably due to the increased number of sleep cycles obtained for analysis 26, 32 and 42 cycles after 0 h, 12 h and 24 h deprivation, respectively

Discussion

The two stages of slow wave sleep responded to sleep deprivation according to predictions The increase in total sleep time following deprivation was due to the increased quantities of DSWS and REM sleep only The quantity of LSWS was unchanged Accordingly, the relative proportion of sleep time spent in LSWS decreased markedly while the proportions both for DSWS and REM sleep increased The changes depended on the length of the deprivation and were most pronounced in the beginning of recovery sleep

Previous reports on total sleep deprivation in the cat are mainly concerned with the effects on REM sleep, SWS is not differentiated Kiyono *et al* (1965) found a large increase in REM sleep in the first hours of recovery sleep after 72 h of sleep deprivation in three cats Vimont Vicary *et al* (1966) in a study on selective REM sleep deprivation, also let a few cats undergo total sleep deprivation They found a relative augmentation of SWS during recovery sleep and no immediate rebound of REM sleep From the present material it is clear that there is an increase in minutes of SWS after deprivation but it is only the DSWS that contribute to this increase

There were no great difficulties in keeping the cats awake They were not allowed to show behavioral signs of drowsiness (Ursin 1968) even though drowsiness was not counted as sleep in the present study Painful stimulation was never used and stress was kept to a minimum This required almost constant interaction with the cat Therefore, it was impossible to monitor the EEG during the deprivation Ferguson and Dement (1967) have reported the same experience

Vimont Vicary *et al* (1966) recording EEG during their sleep deprivation, reported serious difficulties in avoiding cortical spindles In addition to the difficulties in keeping the cats awake when EEG is recorded their findings may be due to the use of a different set of criteria for sleep The cortical spindles they describe may be identical to the bursts of 4—8 c/s high voltage activity that often occurs in cats with open eyes This activity has been referred to as rest rhythm by Hess, Koella and

Akert (1953) and drowsy pattern by Sterman *et al* (1965) and Ursin (1968) None of these authors consider this activity to be sleep However, the Lyon laboratory seems to include the drowsy pattern in their SWS definition (Putkonen and Putkonen 1971). The sleep deprivation used by Vimont-Vicary *et al*, therefore, requiring cortical activation, is not comparable to that used in the present report This is perhaps the main reason for the discrepancy between their data on the one hand and the results of Kiyono *et al* (1965) and the present study on the other

In experiments on restricted sleep, Ferguson and Dement (1967) found an increase in REM sleep per cent of total sleep and a decrease in SWS per cent when sleep was reduced to a few hours per day They did not differentiate between the two stages of slow wave sleep, but Karadžic (1969), using the same method, has identified LSWS and DSWS following the same criteria as in the present report She found that with restricted sleep, LSWS per cent of total sleep decreased, DSWS per cent was not changed, and REM sleep per cent increased

The ratio between REM sleep and DSWS was unchanged after sleep deprivation REM sleep time in minutes was roughly half of DSWS time regardless of preceding deprivation When sleep deprivation is more drastic or when total sleep time is restricted, this ratio may change REM sleep was relatively more increased than DSWS in the experiments on restricted sleep Under such conditions it is possible that intensity changes or other qualitative compensatory mechanisms may occur within each of the different sleep stages The simple comparison of time spent in each stage may then be of little use

The present results are compatible with findings in humans Berger and Oswald (1962) and Williams *et al* (1964) found an increase of deep SWS (stage E or stage 4) during the first recovery night after 108 h and 64 h of sleep deprivation REM sleep was increased the second recovery night Kales *et al* (1970) found increases in both stage 4 and REM sleep on the first recovery night after 205 h of sleep deprivation The light SWS stage (stage C or stage 2) was reduced in all three studies

The selective increases in DSWS and REM sleep after sleep deprivation seem to indicate that the organism needs some aspects of these sleep stages rather than LSWS The absolute quantity of LSWS is not affected by sleep deprivation and it is relatively reduced during postdeprivation sleep Sleep deprivation thus accentuates the functional dissociation between the two slow wave sleep stages described previously (Ursin 1968) It is premature to decide whether this dissociation derives from a real dichotomy into two separate mechanisms It is possible that the findings reflect an intensity change that SWS is a continuum and that DSWS is more effective than LSWS or that certain functional demands are met only by the deeper stages

The earlier findings also indicate a particular relationship between DSWS and REM sleep (Ursin 1968 1970) It was suggested that this relationship comprises two factors A triggering factor, possibly neural was suggested by the fact that DSWS almost always preceded REM sleep A duration control factor, possibly humoral, was suggested by the quantitative relationship of DSWS and REM sleep

between cats and within the sleep of individual cats These two factors are not affected by moderate sleep deprivation

An increase in the number of sleep cycles is a major feature of the postdeprivation sleep There are also indications of slight qualitative changes within the sleep cycles Postdeprivation sleep has relatively fewer and shorter LSWS episodes than non deprivation sleep DSWS episodes are slightly longer and the REM sleep episodes tend to be longer The length of the sleep cycle however is not changed Thus there seems to be a third factor not affected by moderate sleep deprivation regulation of sleep cycle length

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K[illegible]

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Chemical Composition of Basophil Granules from Isolated Rat Mast Cells

By

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Abstract

BERGQVIST U, G SAMUELSSON and B UVNAS *Chemical composition of basophil granules from isolated rat mast cells* Acta physiol scand 1971 83 362—372

A protein fraction was isolated from rat mast cell granules Disc gel electrophoresis and peptide mapping showed this fraction to be a mixture of 1 or 2 main components and 2—3 minor components On ultracentrifugation the protein fraction behaved as an homogeneous material with a molecular weight of 5600 Amino acid analysis showed the presence of all the common amino acids (with the possible exception of tryptophan) High contents of lysine, aspartic acid glutamic acid proline glycine and valine were noted The content of cysteine was remarkably low The isoelectric point of the main component(s) was found to be around 9

Since heparin and histamine *in vitro* form a rather stable complex it has been assumed that mast cell histamine is stored ionically bound to the heparin of the basophil granules (Parrot and Laborde 1951, Amann and Werle 1956, Keller 1958 Sanyal and West 1959 Kuttner 1961 Kobayashi 1962) Since zinc has been found in relatively large amounts in mast cells the Zn^{2+} ion has been ascribed a chelating function in the formation of this heparin-histamine complex (References see page 371) The stability of the heparin histamine *in vitro* complex down to pH 3—2 indicates a binding between the strong acidic groups of heparin — OSO_3 - and $NHSO_3$ - groups — and the primary amino group of the histamine However, mast cell granules do not consist entirely of heparin The other main constituent is a basic protein (Lagunoff *et al* 1964) and a heparin protein complex is believed to form the matrix of the histamine storing granule In this complex the strong acidic groups of heparin may be expected to be attracted to the basic protein groups and thus be unavailable for the binding of histamine

During the course of recent studies concerning the mode of binding and release of histamine in mast cell granules evidence was found which indicated an ionic binding of histamine to carboxyl groups in the granule heparin-protein complex (Åborg, Novotny and Uvnas 1966) Titration experiments suggested that these

carboxyl groups belonged to the protein part of the granule complex (Uvnäs, Åborg and Bergendorff 1970)

Our studies have shown that in their amine storing properties the mast cell granules behave like a weak cation exchange resin Such a simple storage mechanism should suffice to explain the release of histamine from mast cells as a cation exchange when as a consequence of the degranulation process the granules become exposed to the extracellular milieu

To obtain a better understanding of the above observations the composition of the mast cell granules has been investigated with special emphasis on the nature of the basic protein

Methods

Isolation of mast cells

Mast cells were isolated from the abdominal and thoracic cavities of male Sprague-Dawley rats weighing 350—400 g by gradient centrifugation in Ficoll dissolved in isotonic buffered salt solution as described by Thon and Uvnäs (1966)

Isolation of mast cell granules

Mast cells were lysed by suspension in deionized water at pH 7 1 and the granules were isolated by differential centrifugation according to Uvnäs *et al* 1970

Solubility of the granules

For investigations on the influence of temperature pH and salt concentration the rats were injected subcutaneously with 4 mCi $Na_2{}^{35}SO_4$ in 1 ml of physiological saline 48 h later the rats were killed and the mast cells isolated Following rupture of the mast cells the coarse cell debris was removed by centrifugation at 300 × *g* for 10 min and the protein concentration of the supernatant determined Since the low speed centrifugation left the granules in the supernatant the protein value was taken to indicate the granule concentration The volume was adjusted to give a protein concentration of 15—45 µg/ml Granules from 2 00 ml aliquots of the supernatant were collected and suspended in 2 00 ml of the incubation medium After incubation for 15 min the sample was centrifuged at 2700 × *g* at 4° C for 25 min and the concentrations in the supernatant of heparin (as ³⁵S) histamine and protein were determined

The influence of pH was studied at 20° C in 10⁻² M buffers except for pH 1 where 0 1 M HCl was used All buffers were 10⁻² M with respect to Na concentration

The influence of salt concentration on the solubility of the granules was studied at room temperature in 10⁻² M Na phosphate buffer of pH 7 containing various amounts of NaCl With increasing NaCl concentrations the pH decreased somewhat being down to 6 in 2 M NaCl solution

Separation of granule protein and heparin

Dowex 1 X2 (50—100 mesh) in Cl form was treated with 2 M NaOH 2 M HCl and then with water until no Cl ions appeared in the filtrate The treated resin was packed into a column (11 × 150 mm) and equilibrated with 1 M NaCl Granules were dissolved in 1 M NaCl to a concentration corresponding to 100 µg of protein ml 15 ml of this solution was applied to the column which was then eluted with 40 ml 1 M NaCl The eluate was desalted by dialysis against distilled water in Visking 18/32 cellophane bags or by ultrafiltration through Amicon UM 2 filters

Protein was determined according to Lowry *et al* (1951) with pepsin as standard or by measuring UV absorption at 280 nm or 230 nm

Heparin was determined at ³⁵S using a Tri Carb liquid scintillation counter or acid according to Dische (1946), using commercial heparin as standard In some was determined according to Bertolacini and Barney (1957

Histamine was determined according to Shore Burkhalter and Cohn (19[illegible]

Disc gel electrophoresis was performed according to Reisfeld Lewis and but without sample and spacer gels The electrophoresis tubes were silico[illegible]

i fraction was subjected to free electrophoresis in 0 1 M Na_2CO_3 buffers using the apparatus constructed by Hjerten (1970) Runs were made at pHs 10 7 and 9 6 and the isoelectric point was calculated from the mobilities at these pH values

Ultracentrifugation was performed with a Spinco, model E, ultracentrifuge equipped with interference optics The solvent was 0 1 NaCl and the temperature 20° C Sedimentation experiments were run at the maximum speed of the centrifuge (59,780 rpm) and equilibrium studies for 20 hours at 52,640 rpm

Amino acid analysis

Proteins were hydrolysed with constant boiling HCl at 110° C for 24 h as described by Hirs Stein and Moore (1954) Following hydrolysis, the sample was evaporated to dryness *in vacuo* in a rotatory evaporator and the amino acids determined with an automatic amino acid analyzer according to Spackman, Stein and Moore (1958) as modified by Samuelsson (1968)

Peptide maps

sin solu solution ubjected (1969)

Test for sugars

The presence of non nitrogenous sugars was tested by the Winzler orcinol sulphuric acid procedure according to François, Marshall and Neuberger (1962) For amino sugars the method of Cessi and Piliego (1960) was used Sialic acids were tested by the thiobarbituric method described by Warren (1959)

The c [illegible] formed

ATP [illegible]

Prote [illegible] substrate

Inhibition of proteolytic activity

To avoid artificial inhomogeneity of the granule protein used for homogeneity studies and chemical characterization diisopropylfluorophosphate (DFP) was added at a concentration of 10^{-2} M in all preparation steps from the first washing of the free granules up to and including chromatography on Dowex 1-X2 This concentration of DFP completely inhibited the proteolytic activity

Materials

Ficoll, AB Pharmacia Uppsala Sweden

[illegible] AB [illegible] S [illegible] Sweden

[illegible] Norwegian Atomic Energy Institute Kjeller, Norway

[illegible] York, USA

[illegible] Jersey, USA

[illegible] Germany

[illegible] rial Chemical Industries England

[illegible] search Institute of the National Defence Sweden

Glass distilled water was used unless otherwise indicated

Results

Solubility of granules

a. *Influence of temperature*

At 0° in deionized water no heparin (^{35}S activity) was detected in the supernatant and only a small proportion of the protein (about 5 %) went into solution. There was no significant increase in the dissolution of these two components until around 40—50° C when it rapidly increased. The granules were almost completely dissolved at 60—70° C. At 0° C about 30 % of the histamine occurred in the supernatant. No appreciable change in the histamine binding capacity was observed until 50° C when the granules went into solution (Fig. 1 a.)

b. *Influence of pH*

Between pHs 4—9 only insignificant amounts of heparin were found in the supernatant; the protein also showed a low solubility below pH 9. Above pH 9 the granules began to go into solution and were completely dissolved at pH 10—11. The granule histamine binding capacity was low at all pHs in the rather strong buffer concentrations used (10^{-2} M Na^{+}), although 30 % of the histamine still remained in the granules at pHs around 6. (Fig. 1 b.)

c. *Influence of NaCl concentration*

The solubility at pH 7 — as indicated by the appearance of heparin and protein in the supernatant — increased slowly with increasing NaCl concentrations up to 0.5 M. At higher concentrations the solubility of both components rapidly rose, the granules being completely dissolved in 1 M NaCl (Fig. 1 c.). The histamine binding capacity was completely lost at the high salt concentration used.

Separation of protein from heparin

When granules dissolved in 1 M NaCl were chromatographed on Dowex 1 X2 the heparin stayed on the column while a protein fraction passed through with the 1 M NaCl eluant. The recovery of protein was found to be dependent on the granule concentration in the sample. At a concentration corresponding to 100 μg protein/ml the yield was about 80 % of the total protein content of the granules but when the granule concentration was increased to about 250 μg protein/ml or more, only about 40 % of the protein was eluted with 1 M NaCl. At the low granule concentration the rest of the protein was eluted together with the heparin when the eluant was changed to 2 M NaCl (Fig. 2). However, at the higher granule concentration 2 M NaCl eluted only 60 % of the heparin and a further 20 % of the protein. To recover the rest of the protein and heparin the NaCl concentration had to be increased to 4 M.

The heparin retaining capacity of the 11×150 mm column of Dowex 1 X2 was sufficient for isolation of the protein of mast cell granules from at least 15 rats. The yield of granule protein was about 60 μg/rat. The main obstacle in this separation

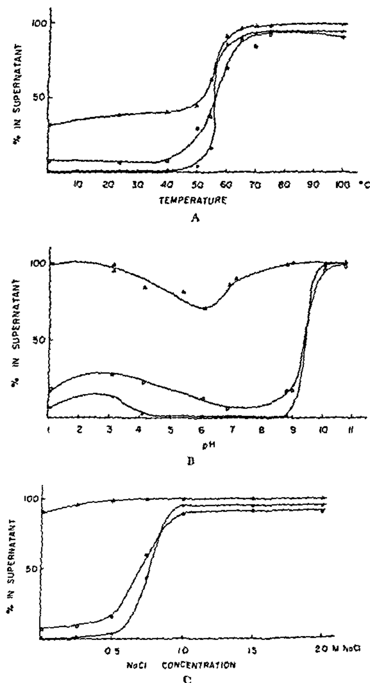


Fig 1 Solubility of *mast cell* granules as a function of

a) *Temperature*
b) pH
c) Sodium chloride *concentration*

●—●—● Heparin (^{35}S)
○—○—○ Protein
▲—▲—▲ Histamine

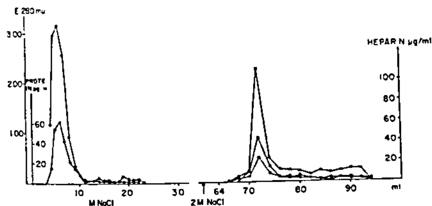


Fig 2 Fractionation on Dowex 1 X2 of protein and heparin from mast cell granules dissolved in 1 M NaCl

●—●—● Protein
O—O—O Protease activity (E 280 mμ)
×—×—× Heparin

procedure was the recovery of the small amounts of protein from the NaCl used for elution Desalting on Sephadex G 25 was found to be impracticable as much protein was adsorbed on the Sephadex even in 5 % HAC or 0 1 M NH_4HCO_3 Dialysis caused the formation of an insoluble protein fraction the amount of which varied from preparation to preparation Best results were obtained by ultrafiltration

Disc gel electrophoresis

On disc gel electrophoresis the protein fraction showed one strong rather broad band and two weaker bands (Fig 3) Some experiments indicated that the broad band might consist of two incompletely resolved bands Minor differences in the number and position of the weaker bands were noted between native protein preparations and granule protein isolated in the presence of DFP Visualization of the two weaker bands required overloading of the disc gel

Isoelectric point

Free electrophoresis of the native protein showed one main and two minor components which all moved towards the anode at pHs 10 7 and 9 6 The mobilities of the 2 minor components were higher than that of the main peak From the mobilities at these two pH values the isoelectric point of the main component was calculated to be about 9

Fig 3 Disc gel electrophoresis of the protein fraction from mast cell granules (not treated with DFP)

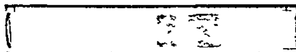


TABLE I Amino acid composition of the DFP treated granule protein (relative mole ratios)[a]

Amino acid	
Lysine	5 17
Histidine	1 84
Arginine	2 87
Aspartic acid	4 00
Threonine	3 38
Serine	2 84
Glutamic acid	4 31
Proline	3 73
Glycine	4 79
Alanine	3 59
½ Cystine	1 48[b]
Valine	4 24
Methionine	0 96
Isoleucine	2 49
Leucine	2 77
Tyrosine	1 90
Phenylalanine	1 55

a) Mean value of two determinations
b) As cysteic acid, single determination

Ultracentrifugation

In ultracentrifugation experiments the granule protein gave only one peak which however was not completely symmetrical The sedimentation constant was $1\,0 \times 10^{-13}$ sec as calculated from 4 exp at different protein concentrations (0 15, 0 31, 0 63 and 0 95 %) and extrapolated to infinite dilution

The equilibrium sedimentation experiments indicated the presence of salts or other low molecular weight impurities The molecular weight for the main component was calculated to be 5600 assuming the specific volume to be 0 75

Chemical composition

The results of amino acid analysis of the granule protein fraction are presented in Table I Neutral sugars were found to be present in an amount corresponding to 2 moles of glucose per 5600 g protein

Peptide maps

According to the amino acid analysis the granule protein contained 5 moles of lysine and 3 moles of arginine per 5600 g of protein Digestion with trypsin could thus theoretically yield 9 peptide fragments if the protein was homogeneous However, about 38 ninhydrin positive spots were found in the peptide map of DFP treated protein Many of these, however were very weak The presence of about 11 strong spots suggsted that the granule protein was a mixture of one main and several minor components, in agreement with the result of the disc gel electrophoresis

The content of Zn

The amount of Zn found in mast cells was 4 1 mμM/10^6 cells and in washed and dried granules 16 2 mμM/mg granules

Content of ATP

The total amount of ATP in mast cells found to be 0 4 μg/10^6 cells This value agrees (well) with data published by Diamant (1967) Only traces of ATP were found in the granules

Content of heparin

The content of heparin determined as $SO_4^{=}$, calculated as heparinic acid in dried mast cell granules was 32 % in agreement with earlier values from Lagunoff *et al* (1964)

Proteolytic activity

4 5 μg of the granule protein digested the same amount of casein in 2 h as 5 μg chymotrypsin under identical conditions Thus on a weight basis the granule protein had almost the same proteolytic activity as crystalline chymotrypsin

Discussion

The granules of rat peritoneal mast cells are insoluble in water and in isotonic salt solution at pH 7 and at room temperature They dissolve in 1 M NaCl solution and can then be separated into two main constituents a basic protein and heparin on Dowex 1 X2 columns

Part of the protein is not eluted from the Dowex column with 1 M NaCl but can be recovered together with heparin at higher salt concentrations The amount of protein more strongly bound to the column is dependent on the concentration of granules applied It is possible therefore that this phenomenon is due to the equilibrium between heparin and the protein At higher concentrations soluble aggregates of heparin and protein might be formed and adsorbed on the anion exchanger The fact that proteolytic activity can still be found associated with the protein in the heparin peak also supports this view (Fig 2)

In addition a minor part of the protein is covalently bound to heparin (Lindahl 1966 Serafini Fracassini Durward and Crawford 1969 Serafini Fracassini Durward and Floreani 1969)

Electrophoretic studies and peptide mapping showed that the granule protein was not completely homogeneous This was also indicated in the amino acid analyses The deviations from whole numbers of amino acid residues were considerable for several amino acids Variations were observed from preparation to preparation. The differences were especially marked for glycine and valine ranging from 5—7 residues for glycine and 2—5 residues for valine Better reproducibility was observed with DFP treated protein desalted by ultrafiltration than with native protein desalted by dialysis

In ultracentrifugation experiments the presence of salts or other low molecular weight impurities was indicated They were later on shown to originate from the cellophane tube used for dialysis of the material

The apparent ultracentrifugational homogeneity of the protein indicates either that the proteins in the mixture were all of the same size or that the concentrations of the minor proteins were low compared to the main components The latter explanation was supported by the results of disc electrophoresis and peptide mapping Ultracentrifugation indicated that the molecular weight was as low as 5600 This does not agree with the figures referred to by Lagunoff (1968), *i e* a molecular weight of 23,000 for the granule protein This discrepancy cannot be explained at the present

The granule protein *showed a high proteolytic activity If it can be shown that* this activity is inherent in the main component of the granule protein this is remarkable bearing in mind the low molecular weight

One reason for the inhomogeneity of the granule protein could be autodigestion during isolation of the protein Although this phenomenon cannot be completely ruled out the fact that preparations made in the presence of DIP were also inhomogeneous indicates that the granules contain several proteins This is in agree
nt with a recent report from Lagunoff Pritzl and Mueller (1970) of the presence
mast cell granules of an N acetyl β glucose aminidase *separable from the prote*
ytic enzyme

Granules isolated either from water lysed mast cells or from cells exposed to compound 48/80 exhibit a considerable chymase activity This might explain the old observation that release of histamine induced either in anaphylaxis or by various other degranulating agents is accompanied by the appearance of protease activity in the blood The appearance of this protease activity has been taken to indicate protease activation as being an essential link in the histamine release process It seems to us more likely that the protease activity observed is secondary to the degranulation and emanates from the granules

The granules have been shown to have the properties of a weak cation exchange material histamine being stored in ionic linkage to carboxyl groups probably localized to the protein in the heparin protein complex (Uvnas *et al* 1970) The histamine binding capacity at pH 7 was found to be around 1000 mμeq/mg dried granules The presence of relatively large amounts of the dicarboxylic acids glutamic and aspartic acid may explain the high cation binding capacity of the complex (see Table I) However the basic character of the polypeptide with its I P around 9 indicates that some of these groups are masked by amidation The exact number of free carboxyl groups will only be revealed by further structural studies

The content of ATP and of phospholipids in the granules is extremely low and excludes these constituent from being of any appreciable importance for the ionic binding of histamine In fact it is doubtful if significant amounts of ATP or phospho lipids occur in the isolated granules

It has been claimed that zinc assists in the binding of histamine by a *chelating*

action (Kerp and Steinhaeuser 1961, Kerp 1963 Pihl and Gustafsson 1967, Angyal and Archer 1968, Fiedler *et al* 1970, Keller and Sorkin 1970) We have found a zinc content in the granules of about 16 mμmoles/mg dried granules This amount is evidently too low to explain any chelating effect of importance in binding 1000 mueq of histamine We did not find an increase in histamine binding capacity of the granules on adding $ZnCl_2$ to a granule suspension On the contrary, any addition of Zn^+ released histamine from the granules as should be expected if histamine was ionically bound In our view it is more likely that zink originates from granule enzymes

We are grateful to Professor S Hjerten University of Uppsala for the performance of the ... were performed by ... niversity of Uppsala ... also due to Professor

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The Distribution of the Yolk Cholesterol in the Young Chick. An Autoradiographic Study

By

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Abstract

SVANBERG, O *The distribution of the yolk cholesterol in the young chick An autoradiographic study* Acta physiol scand 1971 *83* 373—381

^{14}C-4 cholesterol was injected into the invaginated yolks of newly hatched chicks and the distribution of the labelled molecules was recorded by autoradiography ^{14}C was homogeneously distributed in the yolks 1 h after the injection After 4 h, traces of radioactivity were found in the liver and after 12 h ^{14}C had been transported from the liver into most parts of the body The liver content of ^{14}C-labelled cholesterol was very high up to 6 days after the injection The bile was rapidly intensively labelled, and was regularly regurgitated into the gizzard The walls of the large arteries contained significantly more ^{14}C cholesterol than the surrounding tissues Organs engaged in steroid hormone synthesis incorporated more yolk cholesterol than "inactive tissues In the central nervous system of the youngest chicks, radioactivity was only found to any great extent in the pineal gland, while the peripheral nerves were rapidly intensively labelled The retention of ^{14}C-cholesterol after 2 months was greatest in the peripheral nerves and the white matter of the central nervous system Starvation did not markedly influence the relative distribution pattern of labelled material during the first week after hatching

The livers of young chicks contain large amounts of esterified cholesterol 2—3 days after hatching, a decrease of the concentration of cholesterol begins and it reaches the level of adult animals after about 2 weeks (Entenman *et al* 1940, Davison *et al* 1958) The cholesterol in the liver seems to be derived mainly from the yolk and the accumulation rate is highest 2—3 days before hatching (Tsuji *et al* 1955 Davison *et al* 1958, Moore and Doran 1962) The rate of cholesterol biosynthesis in the livers of chick embryos and young chicks is very low up the fifth day after hatching, when it starts to increase rapidly (Goodridge 1968)

The background to the accumulation of cholesterol in the livers of growing chicks is not known and the utilization of the great deposits has so far not been systematically studied.

This investigation was undertaken in order to study the distribution pattern in chicks of the cholesterol deposited in the livers at hatching In a recent report the low degree of utilization of the preformed cholesterol from the yolk in the myelinization process in the nervous system of the chick has been discussed (Svanbe 1970)

Material and methods

Autoradiography was performed on sect ons from Wh te Leah en ch ks n ected h ^{14}C-4 cholesterol into the invaginated yolks
a specific activity of 55 8 mCi/mmol,
England Each chick received a dose
a time at varying intervals, i e 10 and 30 min, 1, 4, 12 and 18 h, and 1, 2, 3, 4, 5, 6 7, 8 9
and 60 days after the injection They
for young chicks and had free access t
by infra red lamps placed in one cor
Bergstrom and Wintersteiner (1941)
Ullberg (1954) has been described els

The films (Ilford, Red Seal 75 FW

and were killed after 4 and 6 days respectively

In the examination of the autoradiographs, the intensity of the blackening was visually estimated The degree of incorporation of ^{14}C into the muscles was used as a reference

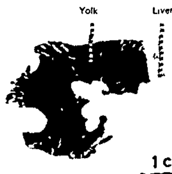
than those involved in growth.

Results

h after the injection of ^{14}C-4 cholesterol, the radioactivity was homogeneously distributed in the whole yolk After 4 h a small amount of ^{14}C had reached the liver, while other tissues showed no radioactivity (Fig 1) In the sections from chicks killed 12 h after the injection, labelled material could be traced in most tissues including the muscles The concentration of the label obviously increased most rapidly in the lungs during the first few hours

From the second day after the injection distinct areas of elevated ^{14}C activity were noted in the superficial parts of the yolk in connection with folds in the yolk sac epithelium (Fig 3 and 4)

The livers of chicks killed between 12 h and 6 days after the injection contained very high concentrations of labelled cholesterol (Fig 2—4), which after the sixth day gradually decreased After 9 days the radioactivity in the livers did not exceed that of the muscles (Fig 5)



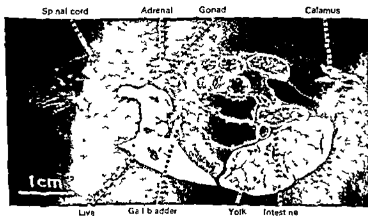
t of a newly hatched ... only in the liver



Fg 2 Autoradiograph of a 1 day-old chick injected with ^{14}C-4 cholesterol on hatching Note the large uptake (white areas) in the liver and in the lining of the gizzard

Radioactivity of the bile in the gall bladders first appeared in chicks killed 12 h after the injection After another 12 h the bile produced as much blackening of the films as the livers and after this time it was by far the most radioactive part of the sections up to the ninth day (Fig 3—5)

The contents of the gastrointestinal tract were radioactive in 12 hour-old chicks not only in the intestine but also in the gizzard The lining of the gizzard showed very prominent radioactivity after 1 day and this condition remained for the rest of the investigation (Fig 2 4 and 5) No radioactivity was found in the contents of the proventriculus (Fig 5) Ligation of the duodenum between the gizzard and the hepatic and cystic ducts before the injection suppressed the appearance of radio activity in the gizzard Except for this the distribution pattern of the radioactivity was the same as that in normal chicks This clearly shows that bile under normal conditions is regularly brought into the gizzard



Fg 3 Autoradiograph of a 4 day old chick injected with ^{14}C 4-cholesterol on hatch ng Note the elevated rad oact v t (white areas) in the superficial parts of the yolk and the differe between the adrenal and the gonad

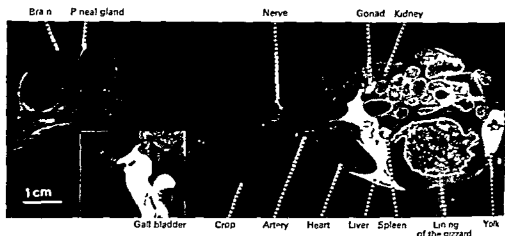
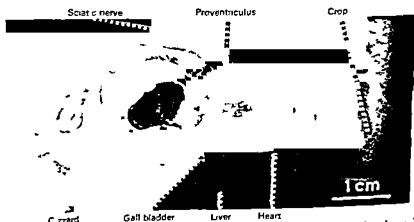


Fig 4 Autoradiograph of a 5-day-old chick injected with ^{14}C-4-cholesterol on hatching Inserted detail from another section Note the intense radiation (white areas) from the gall bladder and the lack of ^{14}C in the central nervous system.

In the 1 day-old chicks the walls of the large elastic arteries had incorporated ^{14}C-cholesterol in larger amounts than the surrounding tissues After 9 days the differ ces were still more accentuated (Fig 6) Two concentric rings of elevated radio- vity was observed one next to the lumen and one in the superficial parts of the all The thickness of the sections made it impossible to recognize the histological structure of the vessels

After the third day the blackening of the films caused by the adrenals was more intense than that caused by the livers indicating a very effective incorporation of cholesterol from the yolk (Fig 3)

Also the gonads incorporated preformed cholesterol but at a slower rate In the 4 day-old chicks the concentration was greater than in the muscles and on the seventh day it was greater than in the livers Fig 3 and 4



... ^{14}C-4-cholesterol on hatching Isotopic ... st extrahepatic tissues The bile ...

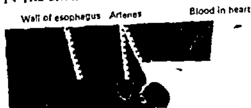




Fig 6 Detail of autoradiograph from the same chick as in Fig 5 Note the high uptake (light areas) in the arterial walls and the very low radioactivity in the blood

Fig 7



Fig 8

Fig 7 Autoradiograph of the head of a chick 2 months after the injection of ^{14}C-4 cholesterol on hatching Note the intense radioactivity (white areas) in the peripheral nerves and the less radioactivity in the white matter of the brain (Exposed for 7 months

Fig 8 Autoradiograph of the head of the same chick as in Fig 7 Note the high uptake (light areas) in the optic lobe

In the central nervous system of the young chicks radioactivity was only found to any noticeable extent in the pineal gland (Fig 4) The peripheral nerves however appeared to incorporate cholesterol from the yolk in large amounts from the second day after the injection (Fig 4) In the 2-month-old chickens the peripheral nerv

were by far the most radioactive structures of the body, as will be seen in the films exposed for 7 months (Fig 7 and 8) The white matter in the brain stem the cerebellum the optic lobes and the olfactory bulbs appeared to be labelled to a much smaller extent while the gray matter was nearly void of ^{14}C and equalled the non nervous tissue (Fig 7 and 8)

The calamus of the feathers especially in the tail was rather heavily labelled from the second day (Fig 3)

Starvation did not influence the relative distribution of cholesterol to any great extent Also in the starved birds, labelled material was found in the gizzard

Discussion

The rapid mixing of the injected mass of ^{14}C-4 cholesterol with the contents in the yolk sac points to a very active mechanical stirring As a result of this the injected ^{14}C labelled cholesterol was available for resorption from the yolk after a short time and the specific activity of the cholesterol resorbed should be constant very soon This was confirmed by small differences in the distribution pattern observed in duplicates *The reproducibility of the present route by which a substance* is administered should be of importance for metabolic studies in chicks in general It is also important to note that by introducing a substance into the yolk a depot effect is achieved which may result in equilibration between different pools of the substance in the body

The elevated ^{14}C activity in the superficial part of the yolk observed from the second day (Fig 3) is apparently due to a slower resorption of cholesterol from the yolk compared with other components This leads to an increasing concentration of cholesterol in the post embryonic yolk (Entenman *et al* 1940) The extent of the *areas with higher* ^{14}C *cholesterol concentrations probably shows the distribution* of enzymes involved in the digestion of the yolk and secreted from the yolk sac membranes (*cf* Romanoff 1960 p 1073)

The rapid accumulation of ^{14}C in the liver resulted in the expected and desired situation and supports the idea that the liver cholesterol is derived from the yolk (Davison *et al* 1958 Noble and Moore 1964)

From the liver the labelled cholesterol was distributed simultaneously and in equal amounts into all tissues in which the known function of cholesterol is to contribute to membrane formation The radioactivity thus reflects the growth of the tissue and the turnover of molecules *already incorporated into the structures* It is obvious that in the 9 day-old chick the great amount of cholesterol in the liver originating from the yolk has been transported to other parts of the body or excreted (Fig 5) The radioactivity still present of about the same intensity as that in the muscles probably mainly emanates from the cholesterol molecules which have been built into the membrane systems of the liver cells

The cholesterol content of the livers of adult animals is rather low in comparison with the amount of bile acids produced (Goodman 1965) This implies that the pool

of cholesterol available for bile acid biosynthesis is rather small and therefore must have a high turnover rate Liver cholesterol equilibrates with plasma cholesterol and indirectly with that of other extrahepatic tissues (Goodman 1965, Danielsson and Tchen 1968) The bile acids in the bile will accordingly be labelled to a decreasing degree, parallel to the decrease in the specific activity of the cholesterol present in the body with the probable exception of that of the nervous system (see below) In the gall bladder a concentration of the bile takes place (Schmidt and Ivy 1937), which leads to the very high radioactivity seen in the autoradiographs (Fig 3—5)

In the 9 day-old chicks in this experiment, the yolks were very small, and it may be concluded that the cholesterol of the yolk is nearly all consumed and has also left the liver The concentration of ^{14}C cholesterol in the blood was also very low (Fig 6) This is in accordance with the chemical analysis first made on yolks, livers and blood by Entenman *et al* (1940) A further need of cholesterol in the body of the chick must be supplied by food and endogen synthesis This conclusion is supported by the results of Goodridge (1968) He demonstrated by *in vitro* experiments that liver slices incorporate ^{14}C from glucose into cholesterol at an increasing rate from the sixth day after hatching

The high radioactivity in the lining of the gizzard and in its contents obviously originated from the bile Regurgitation of the contents of the duodenum into the gizzard has been reported earlier (Sturkie 1965 Dam *et al* 1958), but its functional significance is not clear It appears from the autoradiographs that the food contents in the gizzard are thoroughly mixed with bile during the grinding process that occurs here This may be of importance for the digesive activity in the intestine The bile may, for example, enhance the penetration rate of the water soluble enzymes into the food aggregates in the small intestine

The pH in the gizzards of young chicks during their first few days of life varies between 2 7 and 3 5 and later decreases to about 2 0 (Farner 1942 Dam *et al* 1958) In birds, the main fraction of the bile acids is conjugated with taurine (Buddenbrock 1956, p 357) the conjugates having pK_a values of about 2 5 in the micellar form (Hoffman 1968) This means that a certain percentage of these bile salts will be undissociated in the gizzard and with the exception of taurochenodeoxycholic acid will be poorly solvated (Norman 1955) Any glycine conjugates that may occur having pK_a values around 4 7 in concentrations above the critical micellar concentration will be totally unionized at the entrance into the gizzard and probably precipitated There is thus a great probability that these unionized molecules will be adsorbed to the nonpolar rests in the keratinoid lining of the gizzard Also the dissociated bile salts may due to their anionic properties interact with the positively charged keratin The presence of bile salts in the lining of the gizzard may contribute to the toughness and inertness to mechanical and chemical actions which is characteristic of this structure and is reflected in the high radioactivity observed

The adrenals and the gonads which incorporated more ^{14}C than the muscles are involved in steroid hormone synthesis According to Cook (1958) Mazina (1962) and Goodman (1965) the concentration of cholesterol in these tissues is n

high especially in the adrenal The large uptake of ^{14}C in the endocrine glands verifies that equilibration occurs between the cholesterol of the plasma and that of the glands (Samuels and Eik Nes 1968)

The results presented here fully verify the opinion that preformed cholesterol takes part to a fairly small extent in myelin formation in the central nervous system and that ^{14}C cholesterol, once laid down in myelin persists up to the adult stage (*cf* Davison 1968) It has recently been proposed that this does not reflect metabolic stability of the myelin as formerly believed (Davison *et al* 1959) but that it is due to an effective reutilization of molecules in the glial cells (Rawlins *et al* 1970 Bank and Davison 1970)

The localization of ^{14}C to the white matter in the brains of chicks 2 months after the injection (Fig 7 and 8) is very similar to that found by Kritchevsky and Defendi (1962) under the same conditions It also resembles very much the results obtained by Chevallier and Petit (1966) and Appelgren (1967) from experiments on rats and mice given ^{14}C cholesterol at the adult stage Thus it seems as if a certain percentage of preformed cholesterol can be incorporated into the myelin sheaths whether growing or fully developed

The outstanding radioactivity in the peripheral nerves of the 2 month old chicks that the ^{14}C cholesterol which was deposited there soon after the injection ersists over a long period of time It supports the suggestion that the cells of hwann differ from the oligodendroglial cells with regard to the handling of preformed cholesterol in the myelination process (Svanberg 1970) Thus the relative amount of cholesterol synthesized *in situ* appear to be less in the myelin in the peripheral nerves than in the central nervous system However also in adult mice the incorporation of injected ^{14}C cholesterol into peripheral nerves is more rapid than that into the central nervous system as appears from Appelgren (1967

It has been confirmed in this study that the cholesterol accumulated in the livers of newly hatched chicks is derived from the yolk From the livers it is transported to all tissues of the body where it is used in the growth processes to about the same extent An exception is the central nervous system In endocrine glands in which cholesterol is catabolized there is an accumulation of yolk cholesterol Rather large amounts seem to be eliminated from the body with the bile Utilization of the yolk cholesterol along other routes than those expected has not been found

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Studies on Tetrodotoxin Resistant Action Potentials in Denervated Skeletal Muscle

By

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Abstract

HARRIS, J B and S THESLEFF *Studies on tetrodotoxin resistant action potentials in denervated skeletal muscle* Acta physiol scand 1971 83 382—388

n this study it is shown that action potential generation in rat mouse and cat muscles is [illegible]cked by tetrodotoxin (TTX 10^{-6} M) but that the chronically denervated muscles of these [illegible]es are partially resistant to TTX In denervated rat muscles the development of the TTX [illegible] by the spread [illegible] receptor and [illegible] [illegible]ting that the action potential site and the cholinergic receptor are not identical structures Denervated muscles are also partially resistant to saxitoxin although less so than to TTX

It has been reported that denervated rat skeletal muscles develop action potentials resistant to the action of tetrodotoxin (TTX) and that TTX resistance appears at approximately the same time as extrajunctional cholinergic receptors (Redfern and Thesleff 1971 b) These observations raised several questions and in this communication we have attempted to answer some of them These are 1) Is the resistance to TTX common to other chronically denervated mammalian skeletal muscles? 2) Are action potentials in denervated skeletal muscles similarly resistant to saxitoxin (STX)? 3) What is the relationship if any between extrajunctional cholinergic sensitivity and TTX resistant action potentials?

Methods

Most experiments were carried out using male Wistar rats weighing approximately 200 g Extensor digitorum longus muscles (EDL) and soleus muscles (SOL) were denervated by sectioning either the deep peroneal nerve close to the knee or the tibial nerve in the popliteal space as appropriate Mouse EDL muscles were denervated by sectioning the deep peroneal nerve, and cat tenuissimus muscles (TEN) were denervated by sectioning the motor nerve close to its point of entry into the muscle All denervations were made unilaterally At various

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times after denervation the rat muscles were removed and mounted in a constant flow bath irrigated with an oxygenated fluid (Liley 1956) maintained at pH 7 2—7 4 and 28—29° C Cat and mouse muscles were similarly removed and mounted, but only 6—7 days after denervation

Acetylcholine (ACh) sensitivity of muscle fibres was measured by the technique of ionto-
l Katz 1955) using current pulses of 10—100 msec
, one unit being 1 mV depolarization produced by
All values of ACh sensitivity were corrected to a membrane potential of −65 mV (Katz and Thesleff 1957) Action potentials were generated and recorded using a double microelectrode technique (Redfern and Thesleff 1971 a) Briefly, the current passing electrode, filled with 2 M potassium citrate was inserted into the same fibre as the recording electrode at a separation of 50—100 μm The membrane potential was set by a steady anodal current to a local level of −95 mV, and the action potential triggered by a cathodal pulse of 5 ms duration The action potential was differentiated using an RC circuit to yield rate of rise of potential

To compare ACh sensitivity and action potential generation in the same fibre the ACh pipette was also used as the intracellular current passing electrode The pipette was advanced towards the fibre and the local sensitivity to ACh was recorded The pipette was then inserted into the fibre and used to generate the action potential as previously described

When the effects of drugs on ACh sensitivity and TTX resistant action potentials were studied, 20—30 min was allowed for equilibration to take place

Tetrodotoxin 3 × crystalline was obtained from Sankyo Co Ltd, Tokyo Saxitoxin was kindly provided and standardized by Dr E J Schantz Purified cobra neurotoxin was a gift from Dr D Baker, and dinaphthyldecamethonium mustard was a gift from Dr H P Rang All other drugs used were obtained from usual commercial sources

Results

Effects of TTX on the action potential in mammalian skeletal muscle

Action potentials were blocked by TTX (10^{-6} M) in innervated rat EDL and SOL, mouse EDL and cat TEN muscles However as shown in Fig 1 and Table I, fibres of chronically denervated (6—7 days) muscles in the presence of TTX consistently responded to stimulation with action potentials with overshoot, *i e* which exceeded zero membrane potential

Effects of STX on the action potential in rat skeletal muscle

TTX is obtained from the ovaries of some *Tetraodontidae* fish, and from the eggs of some *Taricha* newts, STX is produced by dinoflagellates such as *Gonyaulax*, which are ingested by many bivalves Although both toxins interfere with the movement of sodium ions across membranes during excitation the two compounds are chemically distinct (Kao 1966) Presumably as a result of the differences in the chemical structure, differences in action between STX and TTX have also been observed Thus action potentials in the desheathed nerves of *Tetraodontidae* and *Taricha* species are blocked by STX while they are almost totally resistant to TTX Furthermore the blocking effect of STX on nerve and muscle is more readily reversed than that of TTX (Kao and Fuhrman 1967) These observations made it of interest to examine the effects of STX on action potentials in denervated skeletal muscle

As shown in Fig 2 the rate of rise of the action potential in innervated rat EDL muscle is depressed by STX in concentration of 10^{-9} M and completely blocked in concentration of 10^{-7} M In denervated muscle (3—7 days) resistance to ST

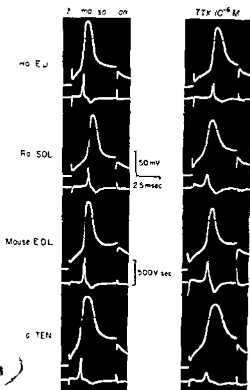


Fig 1 Intracellular recordings of a typical action potential (upper trace) and its first derivative (lower trace) from 6—7 days denervated rat EDL and SOL mouse EDL and cat TEN muscles before (left hand panels) and after (right hand panels) the addition of TTX 10^{-6} M The break in the action potential indicates zero potential in the cell

TABLE I Rate of rise and overshoot of action potentials in various muscles and species before and after the addition of TTX 10^{-6} M The muscles were denervated for 6—7 days The values are means ± S E and the figures within parenthesis give the number of muscle fibres examined

Muscle	Without TTX		With TTX	
	Rate of Rise (V/s)	Overshoot (mV)	Rate of rise (V/s)	Overshoot (mV)
Rat EDL	407 13 2 (36	28 + 1 1 (36)	165 ± 7 9 (45)	16 1 3 (40)
Rat SOL	328 − 12 8 (15)	22 − 2 2 (15)	194 ± 12 1 (28)	9 1 8 (21
Mouse EDL	511 ± 15 6 (40	39 0 9 (40	242 ± 9 7 (35)	29 0 9 (35
Cat TEN	355 ± 22 4 (8	37 3 6 (8	205 − 12 9 (12)	25 2 5 12

seen the action potential being little affected by concentrations lower than 10^{-5} M However as shown in Fig 2 there is not the same resistance to STX as there is to TTX (see also Redfern and Thesleff 1971 b) and regenerative responses are completely blocked in the presence of STX (2×10^{-6} M)

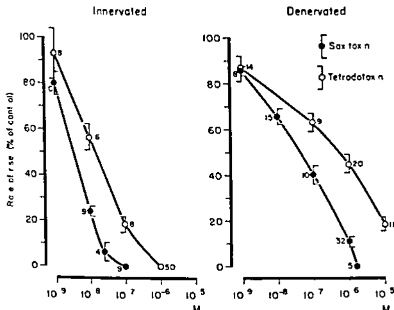


Fig 2 The effects of various concentrations of TTX (open circles) and STX (closed circles) on the rate of rise of action potentials in innervated and 6—7 days denervated rat EDL muscles Means and standard errors are expressed as percentage of the values of the rate of rise in the same muscles in the absence of any drug Figures next to each mean value indicate the number of fibres examined

The relationship between ACh sensitivity and TTX resistance

Temporal aspects The relationship between the duration of denervation and the development of extrajunctional ACh sensitivity and TTX resistant action potentials was examined in rat EDL and SOL muscles Since both ACh sensitivity (Ginetzinsky and Shamarina 1942, Axelsson and Thesleff 1959) and TTX resistance (Redfern and Thesleff 1971 b) appear to develop centrifugally from the end plate region the measurements were made at regions approximately mid way between end plate and tendon All muscles were immersed in TTX (10^{-6} M) By the second day after denervation 90 % of the muscle fibres exhibited TTX resistant action potentials Thereafter the number of fibres resistant to TTX and the rate of rise of the action potential increased By day 3 all fibres were resistant and by day 4 the rate of rise of action potential was maximal *i e* about 60 per cent of control ACh sensitivity, exceeding 0 1 unit was present in approximately 30 % of fibres on day 2 and in all fibres day 4 Sensitivity increased over the whole period of days 2—7 The mean rate of rise of action potential over the first 4 days of denervation was paralleled by the increase in sensitivity to ACh (Fig 3)

Pharmacological aspects The observations concerning the temporal aspects of TTX resistant action potentials and the ACh sensitivity suggested that we should examine the possibility that the TTX resistant action potential generating site w

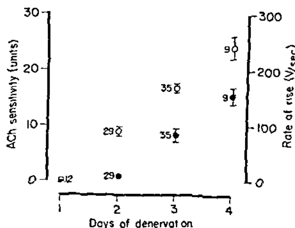


Fig 3 The time course of the development of TTX resistant action potentials and cholinergic sensitivity during the first four days after denervation The mean (± S E) of the rate of rise of action potentials in TTX (10^{-6} M) is plotted on the right hand ordinate and the mean (± S E) of the sensitivity to ACh is plotted on the left hand ordinate Figures next to the means represent number of fibres examined

identical with or closely related to the extrajunctional cholinergic receptor If the two sites were closely related structures, we considered it possible to modify the properties of one by interfering with the other Accordingly, a number of drugs capable of blocking or modifying the response to ACh were studied for their possible effects on the TTX resistant action potential As can be seen in Table II competitive cholinergic blocking agents (tubocurarine, gallamine), irreversible cholinergic blocking agents (dinaphthyldecamethonium mustard Rang and Ritter 1970, purified cobra neurotoxin Eaker Harris and Thesleff 1971) agents blocking the muscarinic respectors (atropine) and anti cholinesterases (physostigmine DFP) were all without effect on the TTX resistant action potential Complementary to

TABLE II Effect of drugs on the ACh response and the TTX resistant action potential in 3 and 7 days denervated rat EDL muscles + – response blocked — response unaffected (< 20 % change)

Drug	Concentration	3 day denervated		7 day denervated	
		ACh Response	Action Potential in TTX 10^{-6} M	ACh Response	Action Potential in TTX 10^{-6} M
Tubocurarine	10^{-5} M	+	—	+	—
Gallamine	10^{-5} M	+	—	+	—
DNM*	2.8×10^{-6} M	+	—	+	—
Cobra neurotoxin	10^{-7} M	+	—	+	—
Atropine	10^{-6} M		—		—
Physostigmine	10^{-6} M		—		—
DFP**	2.5×10^{-6} M		—		—
Saxitoxin	10^{-6} M	—	+	—	+

* Dinaphthyldecamethonium mustard
** Diisopropylfluorophosphate

these observations, it was noted that STX (10^{-5} M), which blocked regenerative responses in denervated muscle, did not modify the response to ACh (mean ACh sensitivity ± S.E. before STX 18.8 ± 8.0 units n = 8, with STX 10^{-5} M 16.0 ± 1.7 units n = 10).

Discussion

The results obtained show that the development of TTX resistant action potentials in chronically denervated muscles is a feature common to various muscles in several mammalian species. It may be common to all species. If this is so, the presence of TTX resistant action potentials is a sign of denervation perhaps as important and specific as the increase in extrajunctional ACh sensitivity.

The inward movement of sodium ions during the action potential in nerve and muscle membranes is specifically blocked by STX and TTX (Kao 1966). Both drugs act in nanomolar concentration, and both are selectively bound to the sites of sodium transfer in the nerve membrane (Cuervo and Adelman 1970, Keynes, Ritchie and Rojas 1971). In spite of the similarity in action however, the drugs are chemically distinct (Kao 1966). Our results show that a denervated muscle is resistant not only to TTX but also to STX. Furthermore it appears that the two drugs can be differentiated on the basis of the sensitivity of denervated skeletal muscle to their blocking effect on the action potential. Thus the action potential in denervated skeletal muscle is considerably more resistant to TTX than to STX.

Since binding in the nerve membrane of both TTX and STX appears to be specific, it is reasonable to suppose that both agents are bound to the membrane structure around the sodium ionophore. If this is so in the muscle fibre membrane, the resistance and the differential sensitivity to TTX and STX following denervation could be the result of a structural change in the membrane surrounding the ionophore, preventing TTX and to a lesser extent STX from reaching its binding site or from blocking the sodium conductance. It is of interest to note that the differential sensitivity of denervated muscle to TTX and to STX is qualitatively similar to the differential sensitivity of the nerves of *Tetradontidae* and *Taricha* species which are also resistant to TTX but blocked by STX (Kao and Fuhrman 1967).

The developmentof TTX resistance in denervated mammalian muscle fibres is closely paralleled by the increase in extrajunctional sensitivity to ACh. Since the appearance of both TTX resistant action potentials and extrajunctional ACh sensitivity is dependant upon protein synthesis (Grampp, Harris and Thesleff 1971), it seemed possible that the active site of the cholinergic receptor protein was the structure which acted as the generating site for the TTX resistant action potential. This now appears unlikely since our experiments on denervated muscle have shown that it is possible with drugs to selectively block either the TTX resistant action potential or the cholinergic receptor. However, the possibility remains that the receptor protein constitutes the hindrance which prevents the access of TTX to the sodium ionophore.

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Properties of Hematopoietic Progenitor Cells

By

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Abstract

Benestad H B and H Breivik *Properties of hematopoietic progenitor cells* Acta physiol scand 1971 *83* 389—398

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in chamber cultures formation of granulocytes and macrophages is prominent whereas erythropoiesis is scant or absent This differentiation pattern was not changed by adding erythropoietin to the culture inocula. Nor did admixture of antigens to the cell inocula influence the formation of granulocytes and macrophages The chamber progenitor cell population as a whole was found to have a near average adhesiveness to glass and fibrin when compared with normal bone marrow cells or blood leucocytes

Index words

Hematopoietic progenitor cells diffusion chamber culture spleen colony forming units granulocytes macrophages erythroblasts immunoblasts, hydroxyurea erythropoietin antigens cell adhesiveness, mouse

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Hematopoietic progenitor cells are the source of differentiated blood cells A study of these progenitor cells may result in better understanding of regulatory mechanisms of cytodifferentiation as well as of mechanisms behind diseases like acute leucemia and aplastic anemia

The progenitor cells are probably heterogeneous in several respects *e g* in cell size and density (Haskill and Moore 1970) in self renewal probability Bennett and Cudkowicz 1968 a) in differentiating potency (Silini Pons and Pozzi 1968 Bennett and Cudkowicz 1968 b) as well as in growth characteristics (Schofield 1970) Several assay methods have been developed for quantitative and qualitative studies of hematopoietic progenitor cells (Hellman *et al* 1970 Lajtha 1969 Diffusion chamber culturing has newly been introduced as such an assay procedure Benestad 1970, Bøyum and Borgstrøm 1970 Breivik, Benestad and Bøyum 1971) It ap

pears that different methods may assay different types or functional aspects of the progenitor cells The classical spleen colony technique will thus mainly detect multipotent progenitor cells (CFUs), whilst *e g* the agar culture method apparently assays progenitor cells, which are functionally more restricted (AFUs) (for references see Breivik *et al* 1971)

The main purpose of the present work was to obtain functional characteristics of *the progenitor cells studied through diffusion chamber culturing Another intention* was to find the relationship between this and other assay systems To this end the proliferative rate of those progenitor cells, which lead to chamber granulopoiesis ('Diffusion chamber progenitor cells' — DCPCs) was examined It is known that the multipotent CFUs of normal mouse bone marrow proliferate slowly, *i e* most of them are in a resting (G_0) state, whereas the functionally more committed AFUs proliferate more rapidly (Iscove, Till and McCulloch 1970) It has been claimed that increased erythropoiesis leads to diminished granulopoiesis as a result of competition for the multipotent progenitor cells (Morley *et al* 1970) Therefore, the pattern of cell differentiation was examined also when the chamber cell inocula contained anemic plasma or different doses of erythropoietin (EP)

Some investigators claim that antigenic stimulation of an animal reduces its umber of multipotent progenitor cells (Ivanyi and Černy 1969) On the other d it has also been found that addition to hematopoietic cell suspensions of some itigens leads to an increase in the number and size of agar colonies formed by such ell suspensions (McNeill 1970 a, b) Different kinds of antigenic stimulation of the diffusion chamber cell populations were accordingly attempted Finally, the adhesiveness of DCPSs to glass beads and to fibrin threads was examined since it has been suggested that progenitor cells are monocytoid cells (Tyler and Everett 1966) Most of these functional tests mentioned were performed with two different hematopoietic cell populations, the one with a very low (blood mononuclear cells), the other with a much higher progenitor cell concentration (bone marrow cells)

Materials and Methods

Mice Male or female (C3H×DBA/2)F_1 mice 7—24 weeks old were used as blood and bone

reagents to facilitate the distinction between immunoblasts and proliferative granulocytes (Benestad 1970).

Spleen colony assay Groups of six mice were irradiated with 950 R from a Mueller x-ray machine (Brenvik *et al* 1971)

Each irradiated mouse received 5 or 7.5×10^4 bone marrow cells in 0.5 ml i.v. within two h of cell removal from the donor mice Irradiated control mice received suspending medium only The recipient mice were killed after eight days The spleens were fixed in Bouin's solution for 24 h placed in 70 % ethanol, and stored in coded vials until the macroscopic surface nodules were counted

Elimination of proliferating cells Hydroxyurea kills cells that are synthesizing DNA, *i.e.* cells in S phase, but it does not damage cells in other stages of the cell cycle (Sinclair 1965). Hydroxyurea (Aldrich, Chemical Co, Milkaukee, Wis, USA) 900 mg/kg was dissolved in saline and given i.p. to bone marrow donors two h before cell sampling This dose is broken

(Morse, Renericca and Stohlman 1969) Saline

lastine (Velbe, Eli Lilly & Co., Indiana, USA) to prospective bone marrow donor mice This drug acts as a mitotic spindle poison and kills cells entering metaphase for several hours after its application A large reduction of bone marrow cells results, followed by rapid regeneration

Erythropoietin (EP) preparations One source of EP was isologous heparin plasma from anemic mice Anemia was produced either by four daily phenylhydrazine injections (about 50 mg/kg), or by bleeding one animal about 0.4 ml from its tail, and then injecting one dose of

anemic mice received 200 mg/kg

This was done to stop erythro-

The hematocrits of blood samples

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aplastic anemia. The dialyzed and lyophilized preparation contained about 1 I U of EP per mg protein (correlated to the International Standard

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ing media and in media containing 20 % rat heparin plasma instead of the usual 15 % mouse plasma. In order to increase the antigenic stimulation 1 ml of undiluted rat plasma was injected i.p. Some of the control mice

ing 10±2 % (mean±S E, with 3.0±1.2 % immuno-

blasts (n=6) in the control cultures

Flagella from Salmonella adelaide (Int Stand Strain No 161, 35 fg) were also used to stimulate blood and bone marrow cell cultures antigenically The bacteria were cultured on a swarm agar (Gard 1938), and the flagella isolated by a method slightly modified from that described by Strachiloff Richter and Mohr (1968) The final suspension of flagella was sterile filtered, its protein concentration was then determined by the method of Lowry *et al* (1951) and the suspension was diluted in a balanced salt solution to a protein concentration of approximately 33 μg/ml It was then kept frozen until used

Chamber cell stimulation was carried out with two different concentrations of flagella (0.025 and 2.1 μg/ml) in the chamber cell suspension Further stimulation was attempted by injecting i.p. on the two days subsequent to chamber implantation suspensions of flagella containing a total of 0.33 or 10 μg of protein respectively

The antigenic potency of the preparation was assessed one week after an i.p. injection of 10 μg flagellin to each of four mice by measuring the agglutinin titers Standard Widal technique was used (Kauffmann 1966) Such titration was performed since no increased immunoblast formation was detected in flagellin stimulated blood cell cultures The geometric means of the agglutinin titers were 1/50 for the H antigens and 1/40 for the 0 antigens No agglutinin activity was found in sera from control mice

The suspension of flagella was kindly provided by Mrs Jorunn Sundar National Institute of Public Health Dept of Bacteriology Oslo, who also titrated the flagellin agglutinins of the mouse sera

Glass bead columns were made according to Plotz and Talal (1967), but with some modifications Gilette Scimitar® sterile disposable syringes were filled to the 7 ml mark with beads with a mean diameter of about 0.6 mm (Reflex Perlen, 31/7, Dragonwerk, Ba

Germany), over just enough glass wool to retain the beads A thin layer of glass wool was also applied to the top of the columns

Prior to use a needle was fitted to the syringe outlet, and the column was filled with 3—4 ml of a culture suspending medium containing a somewhat lower concentration of mouse heparin plasma (10 %) than otherwise used The column was then incubated with this content at 37° C One to two ml of a single cell suspension of bone marrow cells in the same kind of

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1970) was applied to the column The first 4 ml of cell suspension sampled after this application was discarded, the next fractions were pooled and assumed to contain predominantly glass-adherent cells A total of 20 ml of this Ca^{++}- and Mg^{++}-deficient fluid was forced through the column in the course of a few min

About 70 % of the recovered cells belonged to the non-adherent population The procedure provided a relative enrichment only and not a complete separation of adhesive and non-adhesive cells This was evident when the admixture of non adhesive erythrocytes to the two suspensions was measured The erythrocyte percentages of three non adherent fractions of bone marrow cell suspensions, — 63, 56 and 50 % —, were reduced to 17, 14 and 13 % in the corresponding adherent cell fractions

Defibrination of 2—5 ml samples of diluted blood to which 3 6—5 5 × 10⁵ bone marrow

defibrinated blood

Results

Effect of hydroxyurea on bone marrow granulopoietic capacity The number of proliferative granulocytes in four-day cultures depends upon the progenitor cell content of the inoculated bone marrow (Breivik and Benestad 1971) To study the effect of hydroxyurea on progenitor cells marrow cells were sampled 2 h after the injection of either hydroxyurea or saline 4 day cultures were then carried out

The effect of hydroxyurea depended markedly on the age of the animals There was a reduction of granulopoietic capacity to about 30 % for 10 weeks old mice A smaller reduction, to about 85 % was found for mice aged 20—22 weeks (Table I)

Experiments with regenerating bone marrow were used to verify that hydroxyurea kills cells engaged in DNA synthesis On the third day after vinblastine treatment, bone marrow cells, including progenitor cells, are proliferating rapidly (Breivik to be published) Hydroxyurea injections then caused a decrease in bone marrow granulopoietic capacity down to about 10 % of the normal value (Table I)

Effect of hydroxyurea on macrophage precursor cells Hydroxyurea treatment of donor animals caused reduced chamber formation also of macrophages This reduction parallelled roughly the reduced granulopoiesis both in normal and in regenerating bone marrow (Table I)

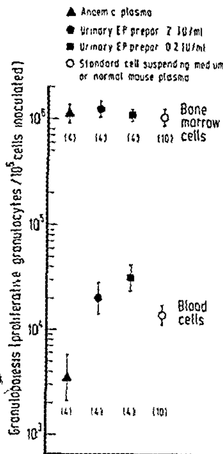
TABLE I Survival of diffusion chamber progenitor cells and spleen colony forming units after hydroxyurea (OHU) treatment of cell donors

Donor mice				Cell harvest from 4-day bone marrow cultures, per cent of inoculated cell number				Spleen colony assay, CFU/10⁵ bone marrow cells	
N	Sex	Age (Wks)	Treatment	Proliferative granulocytes	Survival after OHU treatment (%)	Macrophages	Survival after OHU treatment (%)		Survival after OHU treatment (%)
5	♂	7	OHU	25±6 (8)	19±5	17±3	28±8		
5	♂	7	NaCl	133±16 (8)		61±12			
5	♂	9	OHU	25±4 (9)	27±5	13±2	29±5	25 8±1 8 (5)	79±7
5	♂	9	NaCl	91±9 (9)		45±4		32 7±1 5 (6)	
5	♀	10	OHU	19±4 (9)	30±7	12±2	44±11		
5	♀	10	NaCl	63±5 (9)		27±6			
5	♀	20	OHU	93±14 (6)	73±15	37±6	64±13		
5	♀	20	NaCl	128±18 (7)		58±6			
5	♀	22	OHU	79±11 (7)	99±27	29±6	79±24	20 7±1 7 (10)	98±9
5	♀	22	NaCl	79±18 (6)		36±8		21 1±0 9 (9)	
6	♀	11—14	VLB, OHU	67±18 (9)	11±3	13±3	7±2	61 7±1 5 (22)	33±2
8	♀	11—14	VLB, NaCl	640±50 (9)		170±20		163 ±8 (22	

Hydroxyurea (OHU) [illegible]
cellularity was found 2 h after the OHU injections

Effect of hydroxyurea on bone marrow CFUs In three experiments parallel investigations were performed with the diffusion chamber method and the spleen colony technique With the older mice as donors the number of CFUs was reduced by hydroxyurea to about the same extent as was the formation of granulocytes in the chambers With the younger donor mice and with the vinblastine treated donor mice hydroxyurea reduced spleen colony number to a markedly lesser extent than the granulocyte formation (Table I)

Erythropoietin stimulation of progenitor cells Blood mononuclear and bone marrow cells were cultured for five days with or without EP added to the inocula. Marked stimulation of chamber erythropoiesis was never observed when anemic plasma was used as suspending medium Only once in a bone marrow culture did the erythroblast number exceed 1 % of the chamber cells There was similarly no significant increase in the number of mature erythrocytes in such cultures However a small scale increase in erythropoiesis probably occurred since erythroblasts could easily be found in some of the EP stimulated cultures whilst such cells were very rare in the control cultures The formation of granulocytes and macrophages was not



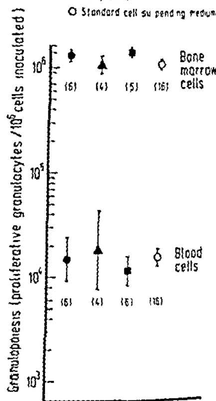


Fig 1 *The effect of erythropoietic stimulation on granulopoiesis in five day bone marrow and blood cell diffusion chamber cultures* The geometric mean number of proliferative granulocytes harvested are shown Standard error and number of chambers are indicated Comparisons between EP stimulated and control cultures were done in eight separate experiments When isologous anemic plasma was used as a source of EP undiluted plasma was used as suspending medium for control cultures The small number of chambers in each experiment and the low concentration of progenitor cells among blood cells are the main reasons for the great SE of some of the data The difference between the control blood cultures and those stimulated by anemic plasma is not statistically significant (P > 0.05 Wilcoxon two-sided test

Fig 2 *The effect of antigenic stimulation on granulopoiesis in five day bone marrow and blood cell diffusion chamber cultures* Comparisons between antigen stimulated and control cultures were done in 16 separate experiments See legend of Fig 1 and text for further explanations

changed by the EP stimulation (Fig 1) The growth in the chambers was not detectably changed from that of standard cultures when pure plasma was used as a cell suspending medium

Similarly, urinary EP, either 2 or 0.2 IU/ml in the cell inocula, failed to stimulate to significant erythropoiesis and to divert progenitor cells from granulopoiesis or macrophagopoiesis (Fig 1)

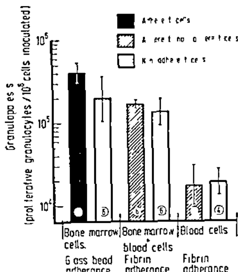


Fig 3 *Adhesiveness of granulocyte progenitor cells to glass beads or fibrin threads* Bone marrow cell suspensions containing isologous plasma and divalent cations were incubated on glass bead columns The non adherent cells were subsequently eluted with the suspending medium The adherent cells were eluted with an EDTA-containing salt solution Between 1 5 and 9 4 × 10 cells were cultured for 5 days

Diluted blood samples some of them containing added bone marrow cells were defibrinated Cells separated from these and from the original intact samples were cultured 2 3—3 2 × 10 cells per chamber for 5 days

Comparisons between adhesive (plus non adhesive) and non adhesive cells were done in 8 separate experiments See legend of Fig 1 and text for further explanations

Antigenic stimulation of progenitor cells The stimulation of chamber cells with rat plasma did not detectably change the formation of granulocytes (Fig 2) or macrophages in blood or bone marrow cultures

The formation of granulocytes (Fig 2) and macrophages was similarly unaffected by the presence of flagella from *Salmonella adelaide*

Glass bead adhesiveness of progenitor cells The results from 4 day cultures showed that granulocyte progenitor cells were apparently not significantly enriched in either the adherent cell or in the non adherent cell fractions (Fig 3)

Fibrin adhesiveness of progenitor cells The granulopoietic capacity of the cell suspensions did not change on defibrination This was found for pure blood cell suspensions as well as for mixtures of blood and bone marrow cells (Fig 3)

Discussion

During the last decades several investigators have attempted to reveal morphological characteristics of hematopoietic progenitor or stem cells However the exact morphology of such cells still remains unknown Our approach has been to get a functional rather than a morphological characterization of the progenitor cells We believe that a functional description is both more useful and also easier to achieve than a purely morphological one

The progenitor cells for the diffusion chamber granulopoiesis (DCPCs) were apparently actively proliferating in the ten weeks old mice of the present investigation Furthermore the proliferative rate of DCPCs exceeded that of spleen colony forming units (CFUs) in all but the oldest mice However we have previously shown that bone marrow CFUs proliferate and increase their number during the early days of diffusion chamber culturing (Breivik *et al* 1971) Such cells might therefore well

be progenitor cells for the chamber granulocytes Other investigators have shown that *CFUs normally proliferate very slowly in situ* (Becker *et al* 1965, Lajtha *et al* 1969), whilst the progenitor cells for agar bone marrow colonies (AFUs) have a higher turnover rate, exceeding that found for DCPCs of older mice in the present study (Lajtha *et al* 1969, Rickard *et al* 1970, Iscove *et al* 1970) The current opinion is that AFUs are committed or *unipotent granulopoietic progenitor cells being per* haps daughter cells of the CFUs (for references, see Breivik *et al* 1971) The present results are therefore probably most easily explained by assuming that the DCPC population is composed of both CFUs and AFUs In the young, growing mice the AFUs would then dominate quantitatively over the CFUs

If the growth fraction of DCPCs is below 1 0, then the degree of reduced granulopoiesis caused by the hydroxyurea treatment does not exactly reflect the reduction of DCPCs (Breivik and Benestad 1971) Furthermore, virtually nothing is known about the conditions for repair of cellular hydroxyurea damage Nor do we know *much about the conditions determining resumption of proliferative activity in the* different assay systems, — the irradiated spleen, the agar dish, or the diffusion chamber Therefore, the data from the hydroxyurea experiments should be inter preted with caution

Erythropoiesis and megakaryopoiesis occur to a very slight degree in standard ...tures The failure of this technique in producing more marked erythropoiesis has been discussed in an earlier paper (Benestad 1970) In the present work the formation of erythrocytes was not substantially increased by EP stimulation of the inoculated cells Nor did the EP influence appear to divert progenitor cells away from the differentiation paths leading to granulocytes and macrophages *It has been* shown by others however that stronger erythropoietic stimulation by 3 large, daily i p injections of EP led to some chamber formation of erythrocytes However, this formation never equalled the granulopoiesis in magnitude (Boyum pers comm) *It has been suggested that the first step of differentiation from a multipotent pro* genitor cell to a committed EP responsive erythroid stem cell is not directly in fluenced by EP (O Grady and Lewis 1970) Perhaps the chamber microenvironment does not favour this differentiation, thus explaining the minimal chamber erythropoiesis

Immunocompent cells and differentiated bone marrow cells have common an cestors (Edwards Miller and Phillips 1970) Antigenic stimulation of chamber cells might mobilize multipotent progenitor cells for immunological tasks thereby di minishing the granulopoiesis Such diversion has been claimed to be a probable *mechanism in in vivo* experiments (Ivanyi and Černy 1969) This hypothesis was not supported by the results of our experiments Some antigens may also play a more direct role for the formation of granulocytes and macrophages Flagellin has thus been reported to increase the number and size of agar bone marrow colonies when added to the inocula in concentrations similar to those used in the present study (McNeill 1970 a b) We found no such effect on the diffusion chamber cultures If such an *in vitro* effect of some antigens represents a physiological progenitor cell

stimulating system, then this might possibly be operating in the diffusion chamber cultures triggered by *e g* antigens absorbed from the guts The presence of additional antigens in the cultures might then not enhance this effect

Hematopoietic cell populations enriched with or depleted of cells adhesive to glass or fibrin remained unaltered with regard to progenitor cell concentration Accordingly, either the progenitor cells have an intermediate degree of adhesiveness to glass or fibrin or the progenitor cell populations were composed of a mixture of adhesive and non adhesive cells The adherance properties of AFUs appear to be unknown Glass wool filtration of mouse bone marrow cells failed to change the concentration of granulopoietic CFUs Erythropoietic progenitor units have, however, been shown to be enriched by glass wool filtration procedures (Bennett and Cudkowicz 1968 b) It would fit our results therefore if multipotent and erythropoietic CFUs have a relatively low adhesiveness, whereas granulopoietic CFUs and AFUs have a higher one

It has been suggested on rather loose grounds that hematopoietic stem cells look like monocytes (Tyler and Everett 1966 Barnes and Loutit 1967) Monocytes are cells which have a high adhesiveness but the results of the present investigation show that at least some of the DCPCs have an intermediate or low adhesiveness However, these findings probably do not allow conclusions to be drawn about progenitor cell morphology since no clear and simple relationship seems to exist between cell adhesiveness and cell morphology (Plotz and Talal 1967)

We wish to thank Miss Kristi Warhuus Miss Wenche Heimli and Mrs Randi Andreassen for excellent technical assistance

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The Influence of Physical Training and Other Factors on the Subjective Rating of Perceived Exertion

By

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Abstract

EKBLOM, B and A N GOLDBARG *The influence of physical training and other factors on the subjective rating of perceived exertion* Acta physiol scand 1971. 83 399—406

The rel[illegible] ceived exertion (RPE) and different [illegible] 19 healthy subjects under the follow- [illegible] perimentally changed during work by [illegible] during different types of physical work [illegible] before and after [illegible] ations [illegible] betwe[illegible] tachy[illegible] was h[illegible] as during bicycling compared to running or swimming A better correlation was found in these experiments between RPE and blood lactate concentration After training, and in parallel to the decrease in HR at submaximal work loads RPE was lower for a given level of oxygen uptake, but was the same when related to the 'relative (per cent of maximum) oxygen uptake

An important question for work physiologists and psychologists is how an individual perceives the amount of work he is performing In 1962 Borg devised a simple rating method in which the strain of a physical work is subjectively evaluated (Borg 1962) A high correlation between heart rate (HR) and the subjective rating of perceived exertion (RPE) has been demonstrated in normal subjects (Borg and Linderholm 1967) as well as in young individuals differing in activity levels and body composition (Skinner *et al* 1969 However, comparisons among different groups revealed differences in perceived exertion, as illustrated by studies on patients with coronary arterial disease and others with vasoregulatory asthenia (Borg and Linderholm 1970)

[1] Dr Goldbarg is on leave from and supported by The Department of Medicine (Cardiol University of Chicago, Chicago, Illin[illegible]

In our laboratory the Borg scale has been frequently used in different studies of the physiological responses to physical work Therefore, it appeared of interest

a) to follow the subjective RPE if HR is experimentally changed during work by the use of autonomic nervous system blocking agents
b) to compare the subjective RPE in different types of physical work, such as bicycling, running, swimming, and
c) to analyze the effects of an 8 week period of physical training program on RPE

Subjects and Methods

19 healthy male subjects aged between 21 and 32 years (mean age 24 years), who performed in one or more of the 3 series of experiments volunteered for this study All subjects were informed about and familiar with the testing procedures Bicycling was done on an electrically braked Krogh or a mechanically braked Monark bicycle ergometer Arm work was done on a mechanically braked ergometer Running took place on a motor driven treadmill The experiments with swimming were done in a specially constructed swimming flume (Astrand and Englesson 1971

In all experiments 2 or 3 different submaximal work loads each of 6 min duration were performed with pauses of about 10 min between each work load Maximal work loads were chosen to exhaust the subjects in 3 to 6 min and were preceded by a 2 to 3 min 'warm up with a load of about 40 to 50 per cent of the individ [illegible] method of choosi[illegible] different days we[illegible] rate (HR) [illegible] loads and continu[illegible] maximal load Blood samples taken from a prewarmed fingertip for determination of blood [illegible] the end of each work load and analyzed [illegible] and Summerson 1941) Oxygen uptake (V[illegible] Collection of air samples was done between [illegible] and the 6th min of each submaximal work load and at least 2 consecutive samples during the last min and a half of maximal work Ventilation was measured with a balanced Tissot spirometer Gas analysis were done with a Haldane apparatus Immediately after the end of each work period the subject was asked to rate his perceived level of exertion (RPE) using Borg scale which is graded from 6 to 20 points (7 corresponds to very very light and 19 to very very heavy)

Experiments with blocking agents The influence of the autonomic nervous system on the oxygen transport system during exercise was investigated in 14 subjects by means of bicycle work (submaximal and maximal loads) during 1) control (C) 2) after parasympathetic blockade (PSB with atropine (2 0—2 5 mg i v) and 3) after betaadrenergic blockade (BAB) with propranolol Inderal®) 10 mg i v) Details of this particular study are presented elsewhere (Ekblom *et al* 1971)

Different work situations Six subjects performed submaximal and maximal work both on the bicycle ergometer and running on the treadmill From this subgroup 3 subjects also performed arm work on the ergometer and 3 other subjects also swam in the swimming flume

Physical training 8 subjects were tested before and after 8 weeks of physical conditioning Training consisted mostly of outdoor cross country running and the subjects trained from 5 to 7 days per week (for the principles of the physical training see Ekblom 1969) Exercise testing was done on the bicycle ergometer and work loads were chosen to demand approximately 25 50 75 and 100 % of the individual s maximal oxygen uptake before and after training respectively In addition after training the subjects performed the work load which was his maximum before the training began

Results

Experiments with blocking agents Maximal oxygen uptake was the same in experiments after injection of 2—2 5 mg of atropine (PSB) or of 10 mg of propranolol (BAB) as in control studies (C with no drugs Maximal heart rate was the same

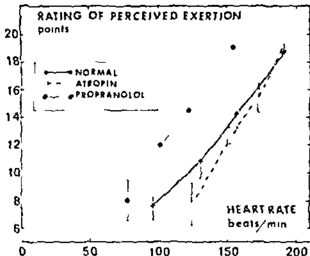


Fig 1 Relationship between perceived exertion and heart rate during bicycle exercise under normal conditions and after blocking the parasympathetic receptors with atropine (2 0—2 5 mg i v) and after blocking the beta adrenergic receptors with propranolol (10 mg i v) Mean values (14 subjects) and standard deviation are shown

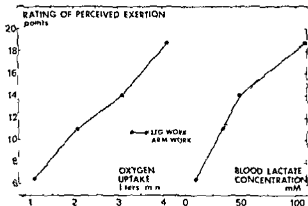


Fig 2 Perceived exertion related to oxygen uptake and blood lactate concentration during arm work and during leg work (3 subjects) Mean values

in PSB as C, but in average 38 beats/min lower after BAB For a given submaximal oxygen uptake PSB caused a higher HR than C For work loads corresponding to 25 per cent, *50 per cent, and 75* per cent of maximal oxygen uptake the increase was 28 15, and 10 beats/min respectively higher than in C After BAB HR for a given submaximal load was lower than C For 25 per cent 50 per cent and 75 per cent of maximal oxygen uptake, HR was 13, 35, and 37 beats/min respectively lower than C

Fig 1 compares the RPE obtained in control experiments to that after PSB and BAB For a given submaximal HR, *e g* 125 beats/min RPE can vary from 8 (PSB) to 15 (BAB) points, compared to 11 points at C RPE during maximal work was the same in all 3 series of experiments For a given submaximal $\dot{V}_{O_2}$ RPE was somewhat higher after PSB and BAB but the differences were not significant Similarly RPE following either PSB or BAB was unchanged compared to control values when related to O_2 deficit, pulmonary ventilation and blood lactate concentration

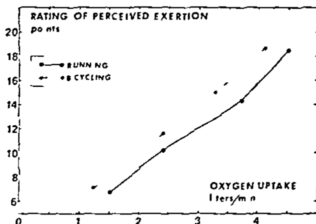


Fig 3 Comparison of perceived exertion between bicycling and running (6 subjects) Mean values

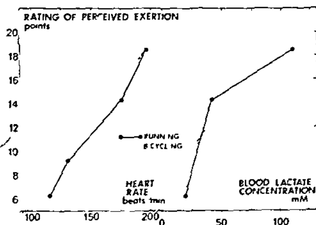


Fig 4 Perceived exertion related to heart rate and blood lactate concentration during bicycling and running (6 subjects) Mean values

Different work situations In Fig 2 RPE in arm work is compared to that during leg work on a bicycle ergometer For a given oxygen uptake RPE in arm work was significantly higher than that of leg work Even for a given submaximal relative oxygen uptake or HR RPE was higher than in arm work However as shown in Fig 2 RPE for a given blood lactate concentration was the same in arm and leg work In Fig 3 RPE in bicycle exercise is compared to that in running When bicycling RPE for a given submaximal $\dot{V}_{O_2}$ was higher than that in running ($p < 0.05$) $\dot{V}_{O_2}$ during maximal work was higher in running than in bicycling — 3 48 l/min and 3 10 l/min respectively So related to the relative load for bicycling and running respectively RPE was the same For a given submaximal HR (Fig 4) RPE was somewhat higher in bicycle exercise $p < 0.05$ At maximum identical values were obtained RPE for a given blood lactate concentration was the same in bicycle and treadmill exercise Fig 4

In Fig 5 RPE in relation to $\dot{V}_{O_2}$ and HR in swimming is compared to that in running For a given submaximal $\dot{V}_{O_2}$ or HR RPE was the same in the two work situations

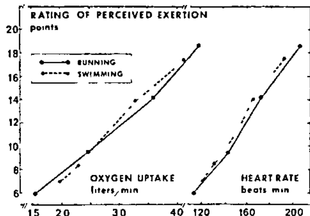


Fig 5 Perceived exertion in relation to oxygen uptake (left) and heart rate (right) during swimming compared to running Mean values obtained from 3 well trained swimmers

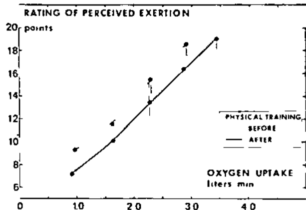


Fig 6 Effects of physical training on perceived exertion Means and standard deviations (8 subjects)

Physical training Fig 6 and Table I summarize data obtained before and after 8 weeks of physical training Maximal V_{O^2} increased from a mean value of 2 90 l/min before to 3 35 l/min after the training For a given submaximal $\dot{V}_{O_2}$ HR was about 15 beats/min lower after the training, compared to before ($p < 0.05$ on all submaximal loads) Maximal HR was unchanged For all standard V_{O_2} at submaximal work loads the rate of perceived exertion (RPE) ws 1 5—2 0 points lower after the training ($p < 0.05$) but the maximum remained unchanged (Fig 6) Related to "relative" oxygen uptake (oxygen uptake in per cent of maximum before and after the training respectively) or HR, RPE was the same before and after the training Oxygen deficit for a given submaximal load after training decreased as compared to pre training levels the maximal values beeing unchanged For a given oxygen deficit and blood lactate concentration, RPE was the same befor[e and] after the training (see Table I)

TABLE I. Oxygen uptake ($\dot{V}_{O_2}$), heart rate (HR), blood lactate concentration (HLa), oxygen deficit (O_2 def), and rate of perceived exertion (RPE) at different submaximal work levels (25, 50, and 75 per cent of pretraining maximal $\dot{V}_{O_2}$) and at maximal work before and after 8 weeks of physical training

work levels	$\dot{V}_{O_2}$ l/min	HR beats/min	HLa mM	O_2 def l	RPE points
25 %					
before	0.96	105.8	19.4	—	9.3
after	0.92	96.5	18.8	—	7.2
p diff	>0.05	<0.05	>0.05	—	<0.05
50 %					
before	1.60	140.3	40.1	0.99	11.6
after	1.61	127.0	29.6	0.85	10.2
p diff	>0.05	<0.05	<0.05	<0.1>0.05	<0.05
75 %					
before	2.24	161.8	66.7	1.72	15.3
after	2.25	150.2	46.8	1.53	13.5
p diff	>0.05	<0.05	<0.05	<0.1>0.05	<0.05
100 %					
before	2.90	191.2	127.3	3.30	18.7
after	3.35	186.7	135.1	3.13	19.2
p diff	<0.05	>0.05	>0.05	>0.05	>0.05

Discussion

In most studies concerning the individual's rate of perceived exertion (RPE), heart rate (HR) was used for the evaluation of the strain of a physical work. In those studies bicycle ergometers were used and a good correlation between RPE and HR was found. The present study shows that such a good correlation is also present when physical work is performed with smaller muscle groups as e.g. arm work (Fig. 2) as well as with larger muscle groups besides bicycling such as running and swimming (Fig. 3 and 5). However, this relationship between HR and RPE can be easily altered in different ways. When the influence of the autonomic nervous system on the circulation is blocked, it can be seen that HR changes markedly for a given oxygen uptake (Ekblom *et al.* 1971). In these experimental conditions the RPE for a given HR — e.g. 125 beats/min — can vary from 8 to 15 points in the scale from 6 up to 20 points after beta adrenergic and parasympathetic receptor blockade respectively (11 points in control experiments — Fig. 1). From those experiments it is quite clear that a tachycardia as such is not the primary factor for the setting of the RPE during exercise. In this study it is confirmed that in many work situations HR mirrors the physical strain subjectively experienced. However, when HR is manipulated by autonomic blockade or influenced by other factors than metabolic ones, such as excitement (Åstrand and Rodahl 1970), after cigarette smoking (Goldbarg *et al.* 1971) etc., work load or oxygen uptake can eventually be used for the evaluation of the physiological strain. This has also previously been stated by Borg

and Dahlstrom in 1962, when studying a patient with atrial fibrillation on a bicycle ergometer (Borg and Dahlstrom 1962)

When comparing bicycle work with running, RPE for a given submaximal oxygen uptake or HR is higher for bicycling than running (Fig 3), but related to the relative oxygen uptake the RPE will be the same in the two work situations It is known, that for a given submaximal oxygen uptake bicycle work will in most subjects cause a higher lactate concentration than running, probably due to a more pronounced static work in bicycling than in running (Hermansen and Saltin 1970) Thus the higher RPE scoring for a given submaximal work on the bicycle may be caused by the higher local muscular strain indicated by the higher blood lactate concentration (see Fig 4)

One of the interesting questions in this connection is however, to find out those physiological factors, which are responsible for the individual's setting of his RPE during exercise From the previous considerations it would appear the individual will evaluate his perceived exertion during physical work due to at least two factors a local factor, *i e* the feeling of strain in the working muscles, and a central factor, *i e* perceived tachycardia, tachypnea, and even dyspnea The importance of the local factors for the RPE is illustrated from the experiments comparing arm and leg work and even from those comparing bicycle work with running But the factors responsible for the RPE are, however, multiple and complex In work with small muscle groups the local factors seem to be dominant, while work with large muscle groups will tend to stress the pulmonary ventilation and the circulation and thus give an addition to the local strain

In the physical training experiments the RPE for a given submaximal load was significantly lower after the training period (Fig 6) This is important to consider since a given work task in daily life activity, such as in manual work, will be perceived easily However, if related to the relative V_{O_2} or HR RPE will be the same before and after the training The explanation for the lower scoring for a given load after training can be found in both the relative lesser strain on the cardio respiratory systems and in the improved function of the working muscles which is reflected in the lower oxygen deficit and blood lactate concentration

In an additional study 3 subjects performed the same maximal work (i with legs only and (ii) with combined arm and leg work (30 per cent of the total load with arms and 70 per cent with leg work) The work time was in average 4 min 30 s with legs only but 5 min 50 s if the same total work was done with arms and legs simultaneously (corresponding work situation has been discussed earlier by Astrand and Rodahl 1970) During the combined work the subjects were asked to score the RPE at the time they otherwise stopped at the isolated leg work For the 3 subjects following figures were noticed (figures for combined work first) 16—19 15—19 16—20 At the end of the combined work the same RPE scoring was noticed as during the maximal works with legs only This shows therefore the importance to perform a given work task with as large muscle mass as possible since RPE du heavy exercise seems to be related to the size of the muscle mass involved,

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Acta physiol. scand. 1971. 83. 407—411

From the Institute of Biological Chemistry, University of Copenhagen, Denmark

The Action of Progesterone Derivatives and Other Steroids on the Sodium Transport of Isolated Frog Skin

By

R W S Tomlinson[1]

Received 14th May 1971

Abstract

Tomlinson R W S *The action of progesterone derivatives and other steroids on the sodium transport of isolated frog skin* Acta physiol scand 1971 *83* 407—411

The ability of certain progesterone deriva [illegible] lated frog skin has been compared relative to pro [illegible] olecular structure reduces or abolishes its ability [illegible] effects to those already reported for aldosteron [illegible] of both progesterone and aldosterone 17B-estradiol produced a stimulation and cortisone, pregnenolone, testoterone and estrone had no obvious effect

In a previous paper it was shown that progesterone when added to the outer bathing medium of the isolated frog skin produced an inhibition of the active sodium transport (Tomlinson 1971) It was suggested that the inhibition was caused by the incorporation of the progesterone molecules into the outer facing membrane with a subsequent blocking of sodium entry This paper reports the results of experiments using derivatives of progesterone and some other steroids to assess the structural requirement of the molecule necessary to produce the inhibition of sodium transport

Methods

The experiments were performed on the ventral abdominal skin of frogs *Rana temporaria* The animals had been stored in a cold room at 4° prior to use

The frogs were double pithed and the dissected skin was rinsed in Ringer s solution The skin was mounted between two glass chambers and the potential and short circuit current measured according to the method of Ussing and Zerahn (1951) and recorded automatically

In the comparative experiments between the degree of inhibition produced by progesterone derivatives and that produced by progesterone itself the skin was divided into two halves One half receiving the progesterone and the other the derivative In the experiments using various other steroids, their effects were compared with a control half skin treated with an equal volume of ethanol

The steroid [illegible]
(16 dehydro [illegible]
(epoxyprogeste
ol 3 20 dione [illegible]

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one), 4-pregnen-17α-ol-3,20-dione (17α-hydroxyprogesterone), 4-pregnen-3,11,20-trione (11-keto-progesterone), 4-pregnen-17α,21-diol-3,11,20-trione (cortisone), 5-pregnen-3β-ol-20-one (pregnenolone), 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol), 4-pregnen-18-al-11β,21-diol-3,20-dione (aldosterone), 4-androsten-17β-ol-3-one (testosterone), 1,3,5(10)-estratrien-3-ol-17-one (estrone), 1,3,5(10)-estratrien-3,17β-diol (17β-estradiol).

The steroids were obtained from Koch Light Co. Ltd., Colnbrook, England, with the exception of aldosterone ("Aldocorten") which was obtained from Ciba Ltd., Switzerland.

The steroids were dissolved in ethanol so that 10 μl of the ethanolic solution when added to the 18 ml of Ringer's solution produced a steroid concentration of 2×10^{-5} M. The steroid was *added to the outer bathing solution of the frog skin.*

Results

The actions of the steroids tested upon the short-circuit current and membrane potential fall into five classes (Fig. 1). Progesterone and its derivatives produce an inhibition within one to two minutes of adding to the outside bathing solution.

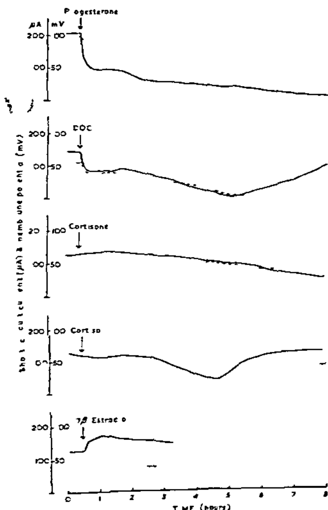


Fig. 1. Representative effects of steroid action on the short-circuit current (———) and potential (- - -) of the isolated frog skin. Steroid concentration was 2×10^{-5} M in Ringer's solution bathing the outside surface of the skin.

TABLE I *A comparison of the inhibitory effects of various steroids at 2×10^{-5} M concentration*

Steroid	Relative inhibition to progesterone	Comments
16-dehydroprogesterone	1 15	
Progesterone	1 00	
4-pregnen 3β 20β diol	0 85	
Epoxyprogesterone	0 51	
11α hydroxyprogesterone	0 49	
DOC	0 45	Moult — latent stimulation
16α methylprogesterone	0 39	
17α hydroxyprogesterone	0 16	
11 ketoprogesterone	0 16	
Cortisone	0	
Pregnenolone	0	
Cortisol	0	Moult — latent stimulation
Aldosterone	0	Moult — latent stimulation
Testosterone	0	
Estrone	0	
17β Estradiol	0	Immediate stimulation (40 %)

Cortisone, pregnenolone, testosterone and estrone produced no obvious effect 17β estradiol produced an immediate stimulation Cortisol and aldosterone after a latent period of several hours produced an inhibitory phase followed by a stimulatory phase Desoxycorticosterone exhibited the effects of both progesterone and cortisol producing an initial inhibition which was followed several hours later by a further inhibitory phase before the final stimulatory phase

The effectiveness of the various steroids in producing inhibition relative to pro gesterone is shown in Table I

Discussion

The inhibition of the short circuit current by progesterone has been described previously (Tomlinson 1971) and the induction of moulting by aldosterone with its characteristic changes in skin potential and short circuit current has been described by Nielsen (1969) It appears that some other steroids share the properties of either progesterone or aldosterone in their effects on frog skin

Cortisol behaves in a similar fashion to aldosterone whilst various derivatives of progesterone have either increased or decreased relative inhibitory capacity depend ing on their molecular modification Desoxycorticosterone possesses the properties of both progesterone and cortisol

The steroid hormones and their metabolites are both water and lipid soluble by virtue of the polar —OH and C—O groups and their sterol framework Willmer (1961) suggested that the specificity of action of a steroid on a cell may depend on its ability to pack into and remain within some surface membrane and the introduc tion of hydrophilic groups of the steroid into the phosph lipid-cholesterol may alter membrane permeability to ions The actions of many steroi

branous structures have in fact been reported Steroids with polar groups at each end but without additional hydroxyl groups in the C11 or C17 position have been shown to alter the permeability of lysosomes (Weissmann 1965), mitochondria (Blecher and White 1960) and erythrocytes (Weissmann and Keiser 1965) The insertion of an —OH or =O group at C11 abolished the lytic effect of a steroid on erythrocytes (Weissmann and Keiser 1965) or on lysosomes (Weissmann 1965) Bangham, Standish and Weissmann (1965) have shown that there is a correlation between the lytic effect of steroids on the release of acid phosphatase from lysosomes and the increase in cation leakage from lecithin/cholesterol/dicetylphosphoric acid swollen structures Steroidal diamines effect the membrane permeability of *E coli* cells and human KB cells growing in suspension but similar effects and greater potency are also found with acyltriamines which have no sterol framework (Silver *et al* 1970)

Progesterone uptake by the membrane of red blood cells is greater than that of corticosterone and cortisol but was thought not to interact with the lipid component but only with a soluble protein fraction of the membrane (Devenuto *et al* 1969) In the toad bladder Porter Bogoroch and Edelman (1964) found that progesterone was diffusely distributed between the nuclear and cytoplasmic areas whereas aldosterone as preferentially associated with the nucleus Nuclear binding sites for aldosterone ave been described for the toad bladder (Edelman Bogoroch and Porter 1963) but n the frog skin the action of aldosterone is also associated with mitochondrion rich cells which may induce desquamation (Voûte *et al* 1969)

On the evidence at hand it would seem that the inhibitory effect of the steroids tested depends on their molecular configuration Small changes relative to progesterone abolishes the inhibitory effect

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Acta physiol scand 1971 83 412—421

From the Department of Pharmacology, Karolinska Institutet Stockholm Sweden

On the Mechanism of Chlorpromazine-Induced Histamine Release from Rat Mast Cells

By

MARIANNE FRISK HOLMBERG

Received 21 May 1971

Abstract

FRISK HOLMBERG M *On the mechanism of chlorpromazine induced histamine release from rat mast cells* Acta physiol scand 1971 83 412—421

On exposure to chlorpromazine in concentrations above 20 μM histamine was released by rat mast cells *in vitro* Histamine release was unaffected by metabolic inhibitors but was reduced by tetrodotoxin elevated extracellular K and Ca concentrations The cellular ratio of Na and K was changed after treatment with chlorpromazine sodium entered and potassium left the mast cells It is concluded that chlorpromazine induced histamine release is secondary to permeability changes in the mast cell membranes and that Na plays an important part in the release mechanism

It is known that chlorpromazine affects the stores of histamine In most species including man histamine is chiefly stored in special tissue cells the mast cells (for references see Selye 1965) In rat peritoneal mast cells histamine has been shown to be stored by an electrochemical linkage to carboxyl groups in the granular protein heparin complex (Uvnas Åborg and Bergendorff 1970) Histamine can be released from these binding sites by ionic exchange with other cations as has been postulated for degranulating agents like compound 48/80 (Thon and Uvnas 1967 and tubocurarine (Frisk Holmberg and Uvnas 1969)

The aim of the present investigation was to study the mechanism of action of chlorpromazine in releasing histamine from rat mast cells The influence of pH temperature time extracellular ion composition and the effect of metabolic inhibitors and tetrodotoxin on release was investigated The intracellular content of Na and K^+ after exposure to chlorpromazine and the uptake of (^{35}S chlorpromazine by mast cells was also determined

Previous studies have shown that chlorpromazine produces one type of response in low doses and the opposite response in high doses (for references see Guth and Sprites 1964) The erythrocyte has been used as a model by Seeman and Weinstein (1966) They found that 1 μM chlorpromazine made the red cell resistant to hypotonic lysis and that higher concentrations (100 μM) produced lysis of the erythrocyte Therefore in this study the effect of chlorpromazine on mast cells exposed to a hypotonic medium was also investigated

Methods

I *Cell preparation*

... '00 g male Sprague Dawley rats were ... Uvnäs 1969), pooled and suspended ... 9 mM $CaCl_2$, 10 % (v/v) Sörensens ... 1 % (w/v) human serum albumin ... solution (BSS) Cell counts were made in a Burker chamber When mixed peritoneal cell suspensions were used, cells were washed twice in BSS pH 6 8 before pooling and suspending

II *Histamine assay* was carried out by the fluorimetric technique of Shore, Burkhalter and Cohn (1959)

Prior extraction of histamine into butanol was not necessary since neither chlorpromazine in the concentrations used nor substances in the mast cell extracts interfered with histamine fluorescence All release values are given either as μg histamine base released or histamine release as a mean percentage of the total ± S D of 6--10 expts The spontaneous histamine release was deducted, unless otherwise noted

III *Incubation techniques*

A. *General procedure*

Unless otherwise stated approximately 100,000 cells were incubated with chlorpromazine in 2 ml BSS pH 7 2 for 10 min at 37° C, followed by immediate cooling (in time course experiments the incubation was terminated by diluting tenfold with icecold BSS) and centrifugation at 4° C for 10 min (400 × g)

Supernatants were decanted and N HCl 10 % (v/v) was added to both supernatants and sediments (adjusted to 2 ml with distilled water) and histamine was determined in both fractions Plastic vials, tubes and pipettes were used as far as possible to eliminate losses due to adsorption to glassware

B *Influence of K^+ and Ca^{++} on release*

The effect on K^+ was obtained by varying the K^+/Na^+ ratio in the incubation medium containing 154 mM NaCl or KCl and 10 % (v/v) Tris buffer 70 mM pH 7 2

Tr ... 154 mM ... were isola... C E... Cell... concentrations of 10 or 100 μM, dinitrophenol (DNP), n-ethylmaleimide (NEM) oubain potassium cyanide or 0 03, 0.3 or 3 μM tetrodotoxin (TTX)

IV *Determination of cellular Na^+ and K^+ content*

About 500 000 isolated mast cells were incubated for 10 min in preweighed tubes with BSS pH 7 2, containing chlorpromazine in concentrations 28—70 μM After the incubation was terminated two washes in 0 34 M sucrose pH 6 9 were performed at 4° C After the final wash tubes were reweighed and thereafter 1 ml of deionized distilled water was added and the cells were frozen and thawed three times Na^+ and K^+ were determined in the samples and washes using an International Laboratories flame photometer model 143 Calculations allowed for the electrolyte content of the remaining drop in the tube Samples for histamine assays were also taken

V *Determination of lactate dehydrogenase (LDH)*

was made in samples containing 30 000—50 000 isolated cells/ml incubation) before during and after incubation 10 min with chlorpromazine (28—70 μM) according to the technique described by Diamant (196... Chlorpromazine in the concentrations used did not interfere with the fluorescence of the reduced form of the pyridine nucleotide (DPNH)

VI *Uptake of (^{35}S) chlorpromazine*

300 000—500 000 isolated cells were incubated in BSS pH 7 2 with (^{35}S)-chlorpromazine 28 to 70 μM for 10 min and thereafter washed 3 times at 4° C in BSS pH 7 2 Final cell sediments were disrupted in distilled water and shaken vigorously centrifuged at 400 × g and samples taken form supernatants for measurement of (^{35}S)-chlorpromazine using a Tri Carb liquid scintillation counter model 3374 The scintillation system consisted of a mixture 2 5-diphenyloxazole, 0 4 % (w/v) and of 1 4 bis 2 (4 methyl 5 pheny...

0.01 % (w/v) in equal volumes of toluene and ethylene glycol monoethylether. The uptake was calculated as μg chlorpromazine base/10^6 mast cells. 40—80 % of (^{35}S) chlorpromazine was washed off during handling.

VII. *Lysis experiments*

About 100,000 mast cells/ml were incubated in 154 mM NaCl 10 % (v/v) 70 mM Tris buffer pH 7.2 containing different concentrations (0.01—10 μM) of chlorpromazine at 25° C for 5 min and centrifuged down. Thereafter 1 ml of 51 mM NaCl and 10 % (v/v) 70 mM Tris buffer pH 7.2 (hypotonic medium) containing identical chlorpromazine concentrations as in the incubation media were added to the mast cells. The controls contained no chlorpromazine. Lysis, as indicated by histamine release, was measured after exposure of mast cells to the hypotonic medium for 15 min at 25° C, and compared to controls.

VIII. *Electron microscopy*

Mixed peritoneal cells from 2—3 rats were incubated for 10 min with histamine releasing concentrations of chlorpromazine in LSS pH 7.2. After terminating the incubation by cooling the cell sediments were resuspended and fixed in 1 % icecold glutaraldehyde in 0.15 M phosphate buffer pH 7.2. Further fixing, embedding, sectioning and examinations were kindly carried out by Dr. T. Hokfelt, Department of Histology, Karolinska Institutet.

Materials

Chlorpromazine hydrochloride and (^{35}S) chlorpromazine (11.4 mCi/mmol) were kindly made available by AB Leo, Helsingborg, Sweden. Human serum albumin, (AB Kabi), and Ficoll (AB Pharmacia) were used. Enzyme inhibitors, tetrodotoxin (Sigma) and all other analytical grade reagents used were obtained from normal commercial sources. Drug solutions were always freshly prepared in deionized distilled water.

Results

Histamine was released from rat mast cells on exposure to chlorpromazine in a concentration dependent manner (Fig. 1). A threefold change of the cell concentration in the incubation did not affect the histamine release as a percentage of the total histamine.

Effects of temperature, time and pH

Histamine release induced by chlorpromazine (28 or 42 μM) was enhanced by an increase in temperature from 0° C to 37° C where the release was approximately 5 times higher than at 0° C. Prewarming cells at 45° C before addition of the releaser did not decrease histamine release. The releasing action of chlorpromazine (in same concentrations as above) started within 30 s and was completed during 5 min. Increasing alkalinization from 6.8 to 7.8 of the incubation medium enhanced histamine release (Fig. 2).

Effects of enzyme inhibitors and TTX

Histamine release caused by 28 μM chlorpromazine was unaffected or slightly potentiated by DNP, potassium cyanide, NEM and ouabain, whereas TTX concentrations of 0.3 or 3 μM reduced release significantly, 25 ± 5.1 % (n = 6) and 40 ± 3.3 % (n = 6) respectively.

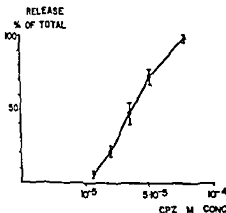


Fig 1

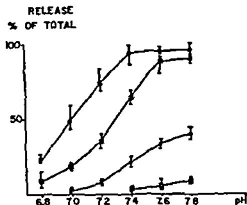


Fig 2

Fig 1 Histamine release fom rat mast cells *in vitro* as a function of the chlorpromazine concentration in the medium Ordinate % of total histamine release Abscissa molar concentration of chlorpromazine Each point represents the mean of 6—7 expt ±S.D

Fig 2 The influence of pH on release of histamine caused by different chlorpromazine concentration 42 ● ● ● 28 ■ ■ ■ 16 ▼-▼-▼ 2 8 □-□-□ μM Ordinate % of total histamine release Abscissa pH of incubation medium Cells incubated in 154 mM sodium potassium phosphate buffer at different pH for 10 min Spontaneous histamine release was less than 5 % and is deducted Each point represents the mean of 6—7 expts ±S.D

Influence of calcium and potassium on release

In concentrations from 1—4 mM, Ca^{++} had an inhibitory effect on chlorpromazine-induced histamine release (Fig 3) Maximal reduction of release was about 50 % at a Ca^{++} concentration of 4 mM Further increase of the Ca^{++} concentration in the incubation medium did not decrease the release of histamine further If K^+ levels in the incubation medium were increased above 15 mM, histamine release decreased At a K^+ concentration of about 40 mM, histamine release was reduced to almost 50 % of control value (Fig 3) Higher K^+ concentrations released more than 20 % of total histamine content

Effect of chlorpromazine on cellular cation (Na and K) and LDH content

The normal mast cell content of Na^+ was 21 1 ± 5 5 (SD) meq/l and of K 105 ± 7 5 (SD) meq/l (n = 8) After exposure to chlorpromazine 28—70 μM the Na content increased and the K^+ content decreased At chlorpromazine concentrations above 50 μM LDH appeared in the incubation medium (Fig 4 At the highest drug concentrations used, 70 μM, the LDH activity in the incubation medium was increased about 50 % compared to controls not exposed to chlorpromazine It should be pointed out that also controls displayed a slight enzyme activity in the incubation medium compared to controls incubated without substrate pyruvate) The LDH dependent oxidation of DPNH, in control cells amounted to 0 28 ± 0 08 (S D) nmol/min (n = 20) This figure agrees with the value 0 1 nmol/min found by

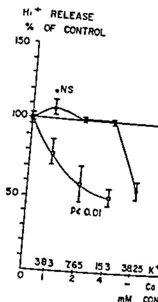


Fig 3 The influence of Ca^{++} O-O-O and K^+ ●-●-● on chlorpromazine (28 μM) induced histamine release at pH 7 2 Ordinate Histamine release in % of control Abscissa concentration of ion tested Values (± S D) (n=6—8) are ... i e release caused of the ion tested ... 11) in a Ca^{++} free medium and 24±1 6 % (n=10) in a K^+ free medium Spontaneous histamine release after K^+ free isolation and suspension 8 7±1 7 % (n=10), and after Ca^{++} free isolation and suspension 9±4 3 % (n=11) $p < 0.01$ when compared to the release in a 1 mM Ca^{++}-incubation

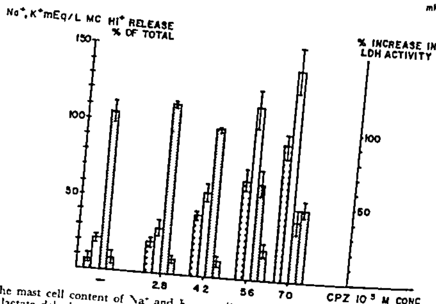


Fig 4 The mast cell content of Na^+ and K^+ meq/l cells % release of histamine and % increase in lactate dehydrogenase activity (LDH) in incubation medium (compared to samples incubated without chlorpromazine) after treatment with chlorpromazine 28—70 μM Values indicate mean ± S D (n=8) The normal mast cell content of Na^+ was 21 1 ± 5 5 meq/l and of K^+ was 105 ± 7 5 meq/l Symbols indicate Histamine release % of total, □ Na^+ content, □ K^+ content, □ % increase of LDH activity in incubation medium

Diamant (1967) This enzyme leakage in the controls specimens was probably due to mechanical destruction of some of the cells during handling

Fig 5 shows the relation between histamine release and Na^+ increase in cells Cellular Na^+ content reaching extracellular Na concentrations when a 100 % of total histamine was released If cells were exposed to TTX 3 μM before addition of

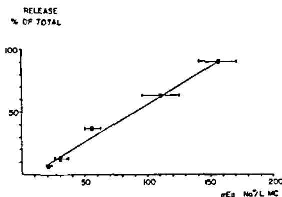


Fig 5 Relation between histamine release expressed as % of total content and mast cell Na⁺ content meq/l cells after treatment with chlorpromazine Ordinate % of total histamine release Abscissa meq Na⁺/l mast cells Values indicate mean ±S.D (n = 6—8)

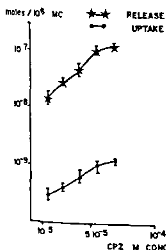


Fig 6

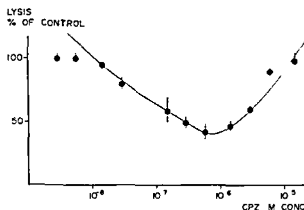


Fig 7

Fig 6 Uptake of chlorpromazine in mast cells ● ● and release of histamine ✦-✦-✦ after incubation with (^{35}S) chlorpromazine Ordinate release or uptake in moles/million mast cells Abscissa molar concentration of chlorpromazine Values are mean ± S D (n = 4)

Fig 7 Effect of chlorpromazine on the hypotonic lysis of mast cells Ordinate % lysis Abscissa molar concentration of chlorpromazine Values mean ±S.D (n = 7) expressed as % reduction in lysis (histamine release) compared to controls only exposed to the hypotonic medium In controls histamine release was 55 + 6 8 % (n 12)

chlorpromazine 42 μM and 28 μM the increase of Na in the cells was reduced by 25 ± 3 4 % (n = 6) and 34 ± 3 5 % (n = 6) respectively if compared to the Na content of cells treated only with chlorpromazine TTX could not decrease the histamine release caused by higher chlorpromazine concentrations The cellular Na increase was one thousandfold greater than histamine release on a molar basis

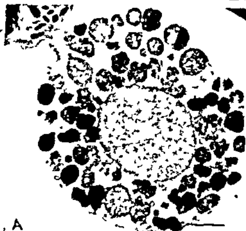
Uptake of (^{35}S) chlorpromazine

The cellular content of (^{35}S) chlorpromazine after exposure to the labelled dru shown in Fig 6 A 100 fold difference between histamine release and









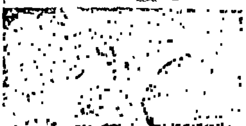


Fig 8—10 Electron micrographs of peritoneal rat mast cells incubated with and without chlorpromazine The mast cells were fixed initially in 1 % glutaraldehyde in phosphate buffer followed by fixation in 4 % glutaraldehyde, postfixed in 1 % osmiumtetroxide, dehydrated in etanol and embedded in Epon Ultrathin sections were cut on an LKB ultratome, contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electronmicroscope Calibration for Fig 10 A—12 A is 5 μ for fig 10 B—12 B 1 μ

Fig 8 A and B Mast cell incubated without chlorpromazine The cytoplasm contains many granules almost all of which have a high electron density Other cell organelles display normal features High power micrograph (B) shows some granules closely surrounded by a perigranular membrane and part of a mitochondrion

Fig 9 A and B Mast cells incubated with chlorpromazine (28 μM) Several granules are swollen and have a low electron density The perigranular membrane of these granules is often disrupted (B) The cell membrane however, appears intact showing many microvilli

Fig 10 A and B Mast cell incubated with chlorpromazine (56 μM) Although the shape of the cell still is relatively unchanged marked alterations can be seen as compared to Fig 10 and 11, almost all granules are swollen and of a low electron density The perigranular membrane is disrupted or absent A cell membrane is only partially present The nucleus is round and of an even, low electron density

(^{35}S) chlorpromazine content was noted Increased pH in the incubation medium did not augment the content of (^{35}S)-chlorpromazine in the cells

Effect of chlorpromazine on hypotonic lysis of the mast cells

Mast cells exposed to the hypotonic medium released 55±6 8 % (n = 12) of their histamine content When the cells were incubated with chlorpromazine concentrations 0 01 to 10 μM before exposure to the hypotonic medium, a decrease in the lytic effect was found (Fig 7) Maximal reduction of lysis about 50 % occurred between chlorpromazine concentrations of 0 1 and 2 μM

Morphological findings

In light microscopy no visible degranulation occurred after chlorpromazine treatment Granules were found extracellularly only when the cells were exposed to chlorpromazine concentrations that released 70—100 % of total histamine content In addition mast cells looked swollen although the cell shape seemed unchanged after treatment with chlorpromazine concentration above 50 μM Electron micrographs (Fig 8—10) show a control mast cells (Fig 8), cells treated with 28 μM (Fig 9) and 56 μM chlorpromazine (Fig 10) giving 20 % and 70 % histamine release respectively

Mast cells treated with chlorpromazine 28 μM (Fig 9) were only slightly changed compared to control cells (Fig 8) The cells nucleus endoplasmatic reticulum mitochondria and microvilli had a normal appearance indicating a fully viable cell, but some of the granules showed structural alterations They were less dense, had increased in size, and in some cases had developed a perigranular space Increased chlorpromazine concentrations affected a greater number of granules and increased the severity of the granular alteration When 70 % of total histamine was released as after treatment with chlorpromazine 56 μM, the whole cell morphology was radically altered The cytoplasm appeared empty, the cell nucleus homogenous and both the cellular and granular membranes were partly dissolved or ruptured (Fig 10)

Discussion

The present results show that chlorpromazine releases histamine from isolated rat mast cells *in vitro* Quantitatively 10—100 % of total mast cell histamine content was released after exposure to chlorpromazine concentrations of 25 μM to 70 μM Greater lipophily of the drug in increasingly alkaline media enhanced release No temperature optimum for release was found Metabolic inhibitors were unable to block release indicating that active metabolic processes are not involved in the release mechanism In agreement with these results both Athee (1966) studying the release of 5 hydroxytryptamine from rabbit platelets and Jansson 1970 studying the release of 5 hydroxytryptamine from a rat peritoneal cell suspension suggested that the chlorpromazine induced release of this amine was not energy dependent In contrast to this histamine release from mast cells caused by degranul-

agents (like tubocurarine and compound 48/80) is sensitive to these physical and chemical factors (Frisk-Holmberg and Uvnäs 1969). Degranulation of mast cells is thought to be trigged by an enzymatic energy yielding process (Thon and Uvnäs 1967) and is blocked by metabolic inhibitors and displays a definite pH and temperature optimum.

The morphological picture after exposure of mast cells to chlorpromazine was also different from the one seen after treatment of cells with degranulating agents (Bloom and Haegermark 1968) in as much as after treatment with chlorpromazine (28 μM) which released 20 % of total histamine content, degranulated cells were not visible. On basis of the morphological findings it is suggested that chlorpromazine-induced histamine release occurs without concomitant granule release unless the cell membrane is partly dissolved or disrupted as after exposure to chlorpromazine concentrations releasing more than 50 % of total histamine content. Then granules are seen extracellularly and Jansson (1970), examining the morphology and 5-hydroxytryptamine release after exposure of mast cells to chlorpromazine, found that *destructive changes occurred in the cells after treatment with chlorpromazine 0.1* mM. Taken together the present data indicate that chlorpromazine induces histamine release from rat mast cells by a mechanism different to that shown for the degranulating agents.

It is known that chlorpromazine alters the permeability in various biological membranes (Guth and Sprites 1964). In this study the cellular levels of Na and K were changed after chlorpromazine treatment. With increasing chlorpromazine concentrations cellular Na increased and above concentrations of 50 μM cellular K content decreased and a gradual increase of LDH in the incubation medium was found. LDH is a cytoplasmic constituent of the mast cell (Diamant 1967). The efflux of this enzyme together with K indicated that a radical change was induced in the permeability of the cell membranes. The present findings that TTX (which prevents Na influx to cells Lane 1968) and Ca (which depresses membrane permeability Manery 1966) opposed the effects of chlorpromazine also indicate that chlorpromazine induced histamine release is the result of an increased permeability in the mast cell membranes. That chlorpromazine protected the mast cells against hypotonic lysis indicating a decreased permeability of the mast cell membrane further supports the hypothesis that chlorpromazine changes the permeability in the mast cell membrane.

The question now arises as to the mechanism of histamine release from granular binding sites. The present findings suggest that Na plays an important role in the liberation of histamine. With increasing histamine release cellular Na content in

[illegible] 100 % the cellular Na content was 150

[illegible] lar levels) and Thon and Uvnäs (1966)

have shown that Na [illegible] emptied histamine loaded granules *in vitro*. The levels of Na found in this investigation are thus sufficient to liberate histamine. That a higher Na concentration was needed for maximal release in this situation might be due to the fact that Na was also bound to other binding sites in

the granules The reducing effect of TTX on cellular Na^+ increase indicates that Na^+ is involved in the intracellular release mechanism It is interesting to note that the release of other amines such as noradrenaline and serotonin from brain slices evoked by electrical stimulation, is depressed by TTX (Katz and Kopin 1969)

The measured uptake of (^{35}S)-chlorpromazine in this study was much lower, about 50 times (when corrected for losses during washes), than the concentration required for histamine release on a molar basis Popova, Slorach and Uvnäs (1970) stated that a chlorpromazine concentration of 0 5 mM was required to empty histamine loaded mast cell granules, but that concentration of chlorpromazine is almost ten times the concentration used to obtain a 100 % histamine release from the cells Therefore it appears unlikely that chlorpromazine participates in the liberation of histamine from granular binding sites, unless concentrations of the drug are used which severly change the cell morphology and the cell membrane function permitting a freely permeable state between the intra and extracellular compartments —a situation when also other cations theoretically could take part in the ion exchange with histamine

This investigation was supported by grants from the Swedish Medical Research Council (B70-14X 39-06B), Ahlen Stiftelsen and Karolinska Institutets forskningsfonder The skilful technical assistance of Miss Ann Katrine Thunfjord is gratefully acknowledged

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Acta physiol. scand. 1971. 83. 422—424
From the Institute of Physiology, University of Oslo, Norway

Separation of Rabbit Blood Platelets for Electronic Particle Counting

By

I AURSNES

At our institute we have been engaged in evaluating the level of circulating blood platelets in rabbits In this connection a method was needed which would allow a large series of platelet counts to be carried out as quickly and accurately as possible It was natural then to attempt an electronic counting procedure This requires separation of blood platelets from erythrocytes in a blood sample A procedure for *uch separation and for subsequent electronic counting of the blood platelets has*, amongst others, been described for human blood by Foss, Rosenlund and Vik (1960) After sedimentation for 2 h of blood which had been diluted 20 times, these authors still found a considerable admixture of erythrocytes in the supernatant They therefore counted these separately and subtracted the number found from *the total corpuscle count in the supernatant In the present series of experiments it* was attempted to adapt this method to rabbit blood and, if possible to improve the separation of platelets from erythrocytes The latter was achieved by selecting a certain and rather moderate height of the blood dilution column The enumeration of the blood platelets with the 'Celloscope", which is not equipped with an upper *threshold devise, is thereby made more accurate and less time consuming*

Results

Initial experiments with rabbit blood were carried out according to the method described by Foss *et al* (1960) The number of erythrocytes remaining in the supernatant was, however high and also somewhat variable Various modifications of the procedure were then tried in order to achieve a better separation It appeared that the height of the column with diluted blood was of crucial importance for the degree of separation seen Short columns gave better separation than long ones, and a column of 7 mm was selected as suitable for the present experiments The following procedure was then developed

Blood The skin over a small ear vein was shaved Silicone grease (Dow Corning) was applied to the field and the vein transsected with a scalpel The first drop of blood was wiped away, and a 50 μl siliconized obstruction pipette was filled from the next drop The blood was immediately transferred to a tube containing 1 05 ml of a 3 1 % filtered citrate solution Mixing was carried out by turning the tube upside down three times

Sedimentation took place in stoppered glass tubes with an inner diameter of 12 mm Enough blood citrate mixture to give a 7 mm high column (as measured from the center of the tube bottom), was added to each tube After 2 hrs the erythrocytes were found packed at the

Phase contrast microscopy was used for direct counting of platelets and cells in the supernatant fluid and for whole blood counts of blood platelets (Brecher and Cronkite 1950, Gruner 1970) At least 500 platelets were included in each counting procedure

In Table I are presented the results from a series of platelet counts carried out in blood from 6 rabbits It can be seen that there is good agreement between results from direct counting with microscope and those from electronic counting The table also shows that the recovery of platelets after sedimentation is good when compared to the platelet numbers found in whole blood counts The scarce admixture of red cells in the sedimentation samples is also given

TABLE I Comparison of platelet counts achieved by direct whole blood counting and by a technique involving sedimentation and subsequent electronic counting Sedimentation was carried out as described in the text Blood from 6 individual rabbits was examined Two electronic countings and two countings in whole blood were carried out for each animal Separate blood samples were used for each of these four countings The two samples for counting in whole blood were taken after one vascular transsection and the two samples for electronic counting after another transsection The results from the individual countings are given in brackets with the mean values above *The electronic counting results are also compared to those achieved from direct microscopy of the supernatant sedimentation fluid*

Rabbit no	Countings from whole blood samples (microscopy), platelets $10^3/\mu l$	Countings in citrate blood mixtures after sedimentation of red cells		
		Electronic counts (platelets) $10^3/\mu l$	Counts by microscopy	
			Platelets $10^3/\mu l$	Red cells in % of platelets
1	373 (352—393)	397 (386—408)	407	1
2	368 (341—395)	383 (378—388)	382	< 1
3	493 (465—520)	476 (468—484)	486	<1
4	458 (445—470)	488 (444—532)	460	6
5	334 (323—344)	374 (352—396)	333	2
6	415 (399—430	460 (458—462)	368	

In order to test the reproducibility of the method ten countings from the same supernatant fluid were also carried out A mean value of 586 000 platelets per μl was found The range was 582 598 000 and the standard deviation ± 5000 platelets

A high degree of accuracy in electronic platelet counting has repeatedly been reported (Foss *et al* 1960, Stormorken Lund Riise and Rorvik 1965) The presented modification of a previously described technique was found to give reliable platelet values within the physiological range in rabbit blood It is easy to carry out and shows accuracy and reproducibility

In our experiments with rabbit blood EDTA was not used as an anticoagulant since for unknown reasons its addition resulted in somewhat variable counts

The present method involves a time delay in achieving a value for blood platelet number, when compared with direct microscopical counting However in research where large series of countings have to be carried out this is probably compensated for by the method being expediant accurate and free from bias

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Acta physiol. scand. 1971. 83. 425—426
From the Department of Clinical Neurophysiology, University Hospital, Uppsala, Sweden

On Muscle Vibration in Man; an Amplitude-Dependent Inhibition, Inversely Related to Muscle Length

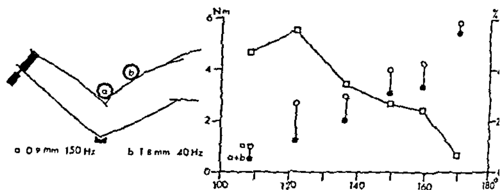
By

G. EKLUND

It has been shown that vibration of certain leg and trunk muscles can influence the balance of a standing man. Bilateral vibration of the calf muscles with 50—160 Hz and a peak-to-peak amplitude of 1.4—1.8 mm caused marked displacements of the common centre of gravity (Eklund 1969). The same vibrators failed to produce such falling reactions if the amplitude was reduced to 0.9 mm, yet, "tendon jerks" appeared at the sudden start of vibration indicating that at least a part of the primary afferents was activated by the stimulus. The vibration induced falling reactions also showed some other characteristics that could not easily be explained in terms of I A reflex effects. In view of these findings it was decided to design experiments to see if there is a corresponding amplitude dependent phenomenon in muscles not engaged in body support and balance reactions.

Tonic vibration reflexes (TVR, Eklund and Hagbarth 1966) were elicited in a comfortably seated subject by vibrating the distal part of the elbow flexors using 150 Hz with an amplitude of 0.9 mm (vibrator "a" in Fig. 1). The arm was securely fastened to a support in which a transducer measured the developed force at the wrist and which could be adjusted to different elbow angles. A second vibrator ("b") with variable frequency and with an amplitude of 1.8 mm was applied over the muscle bellies of the flexor group. Vibrator "a" was switched on by an electric clock for 5 s every 15—20 s (control TVR). In addition vibrator "b" was activated on alternate occasions together with vibrator "a" (compound TVR). It was found that the response to vibration was smaller when both vibrators ran simultaneously provided the "b"-vibrator had frequencies between 25 and 65 Hz. The reduction in strength of the compound TVR was maximal at about 40 Hz and could reach 50 per cent. Above 70 Hz for the "b" vibrator forces greater than control developed. These findings were obtained at an elbow angle of about 120°.

In similar experiments the "b" vibrator was set at 40 Hz while controls and tests were recorded at different elbow angles. The result of such an experiment is shown in Fig. 1. The diminution of the compound TVR compared to the corresponding control-TVR was most prominent at short muscle lengths. Similar reductions in the strength of a voluntary contraction could be brought about with low freq. high amplitude muscle vibration.



TVR filled circles) Left ordinate strength of contraction in Nm for six different muscle lengths (abscissa, elbow angle) The difference in per cent between the control TVR and its corresponding test is represented by the joined squares (right ordinate) The forces at about 110° were small the calculated inhibition (%) is therefore subject to the greatest error

The present findings are consistent with the possibility that the b vibrator excited a length sensitive receptor population with inhibitory properties Granit has postulated (1967) that the group II afferents could provide a length sensitive inhibitory function at least in extensor (antigravity) muscles More experiments are however needed to find out what process is responsible for the observed inhibition

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Acta Physiol. scand. 1971, 83, 427—429

From the Department of Pharmacology, University of Lund, Sweden

OPT-Induced Fluorescence of Glucagon and Secretin

By

R. HÅKANSON, H. JOHANSSON and A. L. [illegible]

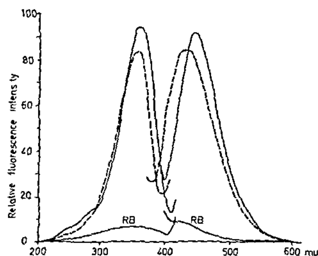


Fig 1 Excitation (left) and emission (right) spectra of the OPT induced fluorophores of glucagon (———) and secretin (- - - -) Concentration of the peptides 1 μg/ml RB Reagent blank Spectra are reproduced as recorded

Ovine thyrotrophin (TSH) was obtained from the Endocrinology Study Section, NIAMD, Bethesda Md Thyrotrophin releasing hormone (TRH) was purchased from Kabi, Stockholm Sweden

The procedure was as follows Small amounts (0 2—25 μg) of porcine glucagon or secretin were added together with 0 1 ml of freshly prepared (recrystallized) OPT (0 2 %) in methanol to 2 5 ml 0 1 N sodium hydroxide at room temperature After 20 min 0 1 ml N citric acid was added The fluorescence was maximal and stable 20—30 min later and was read in an Aminco Bowman spectrophotofluorometer equipped with an x-y recorder Spectral properties of the OPT induced fluorophores of glucagon and secretin are given in Fig 1 Glucagon and secretin gave approximately the same OPT induced fluorescence intensity as equimolar amounts of histamine treated identically (and read at 350/450 mμ) Histidine gave low fluorescence under these conditions Glucagon gave a fluorescence intensity more than twice that of the blank (reaction mixture minus glucagon) at a concentration of 0 2 μg per ml, the fluorescence intensity was proportional to the glucagon concentration over the range 0 2 to 3 μg per ml For the assay of glucagon and secretin in blood or tissues the fluorometric method although sensitive, is probably not specific enough It may be pointed out however that the findings reported may have fluorescence histochemical significance The glucagon storing cells of the pancreatic islets have been found to contain an unidentified histochemically demonstrable OPT reactive component (Ehinger *et al* 1968 Takaya 1970 Håkanson *et al* 1971 In the light of the present findings the suggestion is offered that this OPT-reactive fluorogenic component is in fact glucagon

Grant support from the Swedish Medical Research Council No 72 14X 1007 07) and Albert Påhlsson s Foundation

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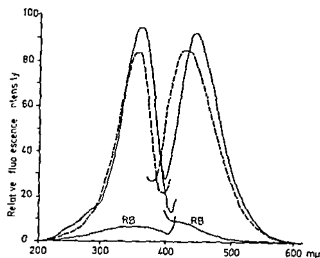


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Grant support from the Swedish Medical Research Council (No 72 14X 1007 07) and Albert Påhlsson s Foundation

(1968) The presence of PG was documented as follows After purification with heptane and extraction with ethyl acetate, perfusate collected during and 3 min after a stimulation period caused contraction of rat stomach strips The contracting prop erty persisted after addition to the incubation medium of atropine (0 1 μg/ml) and 0 2 μg/ml of each of phentolamine, propranolol, methysergide and mepyramine In the guinea pig vas deferens the extract inhibited the neuromuscular transmission, an action claimed to be specific for PG.s of the E type (Hedqvist and Euler 1971) When subjected to thin layer chromatography using the AI and AII systems (Green and Samuelsson 1964), the active factor in the extract cochromatographed with PGE and PGE_2 respectively No PG activity was obtained in perfusates collected during resting periods

Infusion into the spleen of ETA (1 7—3 4×10^{-6} M) 2 min before and during a stimulation period increased the amplitude and/or the duration of the pressor re sponse to nerve stimulation (Fig 1) The outflow of fluorimetrically determined NA was increased by 108±12 % S E M, n = 4, while the efflux of PG was completely abolished The facilitation of the neuromuscular transmission persisted virtually unchanged when the nerve stimulation was resumed 15 min later in the absence of ETA (Fig 1) Doses of ETA below and above 1 7—3 4×10^{-6} M were less efficient and at concentrations beyond 1 7×10^{-5} M the drug actually depressed the NA out flow and the pressor response to nerve stimulation

ETA did not alter removal of ^{3}H-dl NA infused into the unstimulated spleen In fusion of PGE_2 6×10^{-7} M markedly reduced the outflow of NA in response to nerve stimulation (*cf* Hedqvist 1970) In this case ETA did not alter the efflux of NA

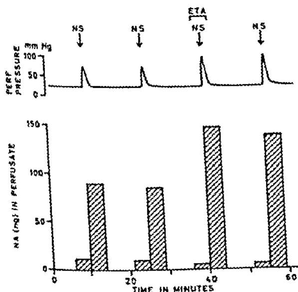


Fig 1 Perfused cat spleen Lower panel Outflow of fluorimetrically determined NA from the spleen resting and in response to nerve stimulation (300 pulses at 10/s) Upper panel Perfusion pressure and pressor responses to nerve stimulation Effect of ETA 3 4×10^{-6} M

Comment

In the present study, nerve stimulation was found to cause the release from the spleen of PG like material, tentatively identified as PGE₂. After administration of ETA the NA overflow and effector responses to nerve stimulation were increased while the release of PG was inhibited suggesting that ETA facilitates the sympathetic neurotransmission in the spleen by inhibition of PG synthesis A direct stimulating action on the NA release process is unlikely since ETA did not effect the resting outflow of NA and since it did not alter the NA overflow response to nerve stimulation when exogenous PGE_2 was continuously infused into the spleen The unchanged removal of ^{3}H dl-NA infused into the spleen in the presence of ETA seems to rule out an action on the NA recapture mechanism

Recent observations indicate that ETA by the same mechanism facilitates sympathetic neuroeffector transmission in the rabbit heart (Samuelsson and Wennmalm 1971)

These observations provide strong support to the view that endogenous PG s of the E type modulate the effector responses to nerve activity in sympathetically innervated tissues (Hedqvist 1970)

This study was supported by grants from the Swedish Medical Research Council projects no B72 14X 3027-03 and B72 14X 3186 02 and from Karolinska Institutets fonder ETA was kindly given by Prof B Samuelsson Stockholm Sweden and PGE_2 by Dr J Pike, Upjohn Kalamazoo U.S.A

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l f r cannulation This vessel should not be freed of all surrounding fat and ce this may induce spastic contraction of the vessel A 4—0 suture of arterial ely placed around the lymphatic, being kept about 2—3 mm away from the rs (Fig 1 b)

n is performed with a specially designed needle attached to a luer-fitting, flexible tubing (St. Thomas' pattern lymphangiography cannula, Macarthys Ltd , Romford, The cannula is checked for patency and lymph clotting prevented by flushing with The tubing is arranged in a loop and hooked around the tip of a clamp attached to n l margin so as to allow minor movements but avoid stretching The needle is grasped a fine-tipped needle holder and slowly advanced into the lymph vessel, which usually is d more superficial in the tissue than one would expect When the needle enters the n dye-stained lymph

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ly gentle traction, prevent blocking of the beveled needle by its lying against the vessel wall Fig 1 c) In the majority of experiments, slight manipulation of these 4 traction sutures will establish a constant flow of lymph through the tubing This is further enhanced by gravity drainage achieved by placing the collecting end of the tubing below the level of the operating table We have
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Fig 2

Discussion

present method of lymph drainage has proved reliable and efficient in more 70 animal experiments Steady and constant lymph flow has been obtained for less no more than 6 h on several occasions Dissection and cannulation is but does not require special instruments other than the cannula rmed by one surgeon without assistance Dissecting our experiments

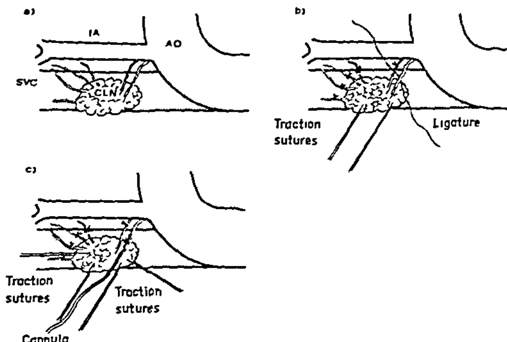


Fig 1 Steps in cannulation of cardiac lymphatic

The cardiac lymph node (CLN) is situated in the grove between the innominate artery (IA) superior vena cava (SVC) and the aortic arch (AO) Two larger afferent lymphatics and several smaller efferent vessels are depicted

b) Traction sutures are applied to the lymph node and all lymphatics are ligated except the one for cannulation The latter is encircled with a ligature

c) The needle is introduced into the lymphatic and lymph is flowing through the tubing The needle is kept in position by tying the ligature Two additional traction sutures are inserted to facilitate positioning of the needle

Method

The approach in the dog is through a left thoracotomy in the IVth intercostal space The lung is retracted posteriorly and the pericardium incised anterior to the phrenic nerve The thymus is dissected away from the mediastinal pleura and displaced with traction sutures The innominate artery is identified and encircled with wet umbilical tape then retracted posteriorly to facilitate access to the "cardiac" lymph node which is situated between the artery and the superior vena cava This gland is the main recipient of cardiac lymph in the majority of animals

Small amounts of T 1824 dye Evans Blue (0 2—0 3 ml) are then injected into the myocardium of the left and right ventricles just beneath the epicardial surface Injection is performed with a no 27 needle and the area screened off with sponges so as to avoid contamination of the pericardium from where lymph is drained along other pathways [illegible] vessels become stained The [illegible] utiously cauterizing all bleed[illegible] g situated in the groove between the two vessels [illegible] ted usually larger than the efferents the latter often being small and irregular At this point 2 traction sutures of 3—0 arterial silk are applied to the gland which is retracted anteriorly to facilitate exposure All visibly stained vessels are then carefully ligated with 4—0 silk except the afferent lymphatic



Fig 2 Drainage of cardiac lymph in a dog Note traction sutures in structures surrounding the cannula. The cannula (single arrow) is in place inside an afferent lymphatic to the cardiac lymph node (CLN), and dye-stained lymph is rising in the tubing (double arrow) Superior vena cava (SVC), thymus (T), heart (H), right lung (L)

which is prepared for cannulation This vessel should not be freed of all surrounding fat and fibrous tissue, since this may induce spastic contraction of the vessel A 4—0 suture of arterial silk is now loosely placed around the lymphatic, being kept about 2—3 mm away from the vessel

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effec of further advancing the needle Two more sutures placed on either side of cannula will, by gentle traction, prevent blocking of the beveled needle by its lying against the vessel wall (Fig 1 c) In the majority of experiments slight manipulation of these 4 traction sutures will establish a constant flow of lymph through the tubing This is further enhanced by gravity drainage, achieved by placing the collecting end of the tubing below the level of the operating table We have also found it helpful to secure the tubing with tape in order to prevent accidental dislodgement of the cannula while performing additional procedures

A picture of the cannula in place and of the surrounding anatomic structures is given in Fig 2

Discussion

The present method of lymph drainage has proved reliable and efficient in more than 70 animal experiments Steady and constant lymph flow has been obtained for periods lasting more than 6 h on several occasions Dissection and cannulation is time-consuming but does not require special instruments other than the cannula The procedure can be performed by one surgeon without assistance Dissecting microscope has not been used in our experiments

Block of the tubing is a rare complication if heparin has been flushed through the cannula and when T 1824 dye is used for visualization of the lymphatics India ink with its larger particles, may block the cannula and is not recommended

In order to ensure that all of the cardiac lymph is collected it is mandatory to look for anatomic variations in the lymphatic pattern (Ullal and Kluge, in press) and to ligate all other channels than the one to be cannulated Detection of aberrant cardiac lymph drainage is ensured by the use of T 1824 dye, whose particles bind to the albumin molecules of lymph and give a distinct staining of all lymphatics draining the areas of injection The validity of this method was extensively tested by repeated injections in numerous areas of the anterior and posterior surfaces of the heart The obstruction of all but one lymphatic channel may account for our values of lymph flow being higher than those previously obtained (Drinker *et al* 1940 Miller 1963)

The presented technique may prove a useful scientific tool in a number of experimental situations No investigations have so far been reported on the contents of lipids, protein components, enzymes, lactate etc in cardiac lymph, such studies may be of considerable interest Investigations on flow and composition of cardiac lymph in anoxia and during extracorporeal circulation are in progress (Ullal and Kluge in preparation)

Recently attention has been drawn to the possible role of impaired lymphatic function in cardiac disease (Miller 1968 Symbas *et al* 1969) Lymphatic drainage from the heart is also necessarily impaired subsequent to cardiac transplantation (Willman *et al* 1962) It thus appears that extensive studies on cardiac lymph may be of importance also to the cardiologist and cardiac surgeon

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Relationship between Antral Acidity and Gastrin Releasing Potency of Chemical Stimulants

By

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Received 23 April 1971

Abstract

ANDERSSON, S and C-E ELWIN *Relationship between antral acidity and gastrin releasing potency of chemical stimulants* Acta physiol scand 1971 *83* 437—445

The effect of antral acidity on gastrin releasing potency of some compounds was studied in Pavlov and H[illegible] compound [illegible] e, acetyl [illegible] pronounced [illegible] 7 in the dose response curve indicating absorption through the antral mucous membrane Absorption of choline showed pH dependency and no signs of cholinergic excitation were obtained at pH 5 or below At low pH levels (pH 1—2) acid may produce a general, physiological insensitivity of the gastrin rele[illegible] of acid most [illegible] responses at pH 5 exceeded the maximal histamine response Responses to acetylcholine and glycine were 60—65 % of maximal histamine response at the most whereas acid output to antral irrigation with ethanol only reached 1/3 of the maximal histamine response

In previous studies (*e g* Elwin and Uvnas 1966) it was shown that various chemical stimuli with different physico-chemical properties release gastrin on perfusion through isolated antral pouches The present study is concerned with the inhibitory action of antral acid pH on gastrin release produced by acetylcholine choline ethanol and glycine

Methods

Surgical procedures

Adult dogs weighing between 10 and 19 kg were used 6 dogs were provided with Pavlov pouches and 6 dogs with denervated fundic pouches of the Heidenhain type About two weeks after construction of the fundic pouches all dogs were provided with an antral pouch with the pyloric end of the antrum exteriorized as a cutaneous fistula on the abdominal surface gastrointestinal passage was restored by gastrojejunostomy Initially all dogs had vagally

vated antral pouches but due to the occurrence of a pronounced spontaneous basal secretion in one Pavlov dog and in one Heidenhain dog the antral pouch was separated from the main stomach by cutting through the muscular layers at the antrum corpus border After this the

regularly For a more detailed description of various surgical procedures is referred to Elwin (1969 a)

Test procedures

The animals had been fasting for about 18 h before start of the experiments Basal secretion from the fundic pouches was determined for at least 1 h prior to the tests Samples of gastric

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id leakage from the

liquid paraffin on top of the test solutions in a vertical open ended glasstube connected to the outflow plastic tube In another type of experiments — hereafter called perfusion experiments — test solutions were perfused through the antrum at a rate of 100 ml/h Periods of perfusion lasted 30 min to 2 h In dose response experiments the antral pouch was continuously perfused with a test solution of one concentration for 1 1/2 h before proceeding to the next higher concentration In dose response experiments with ethanol the perfusion time was limited to 30 min and the secretory response allowed to return to control levels between each concentration

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achieve a pH of 8 or 9 In some dogs maximal histamine response was determined by iv infusion of graded doses of histamine dihydrochloride (0 25—8 0 mg/h) 3 expts were performed in each dog and mean acid output calculated from the last two 15 min periods at each dose level Maximal response occurred with 4 or 8 mg/h

Secretory response to 2 h antral perfusion is expressed as the 3 h acid output from the fundic pouch and to 3 h antral instillation as the 4 h acid output from the fundic pouch In dose response experiments acid secretory output is calculated as the mean values of acid output during the last two 15 min periods of perfusion with each concentration of test agents With ethanol where the perfusion period was limited to 30 min mean values of acid output were calculated from acid secretory output during the two 15 min periods following completion of perfusion at each concentration

Choline and acetylcholine were given as hydrochlorides and doses in tables and figures are expressed as this salt

Results

Antral irrigation with choline

In 20 expts on 3 Pavlov and 2 Heidenhain pouch dogs the antral pouch was perfused with solutions of 1 2 4 and 8 % choline chloride at pH 1 3 5 and 7 (Fig 1 and 2) Except at pH 1 clear dose response curves could be obtained within various concentration ranges No difference of the secretory pattern was found whether or not the antral pouch was denervated With 8 % choline solutions of pH 7 all dogs showed regularly typical signs of general cholinergic effects e g frequent vomiting

[1] Composition of Mac Ilvaine's standard buffer solutions pH 2 2—7 2) Stock solution A 0 1 M citric acid solution and stock solution B 0 2 M disodium phosphate solution

[2] Composition of Tris buffer solution pH 7 2 9 1 0 2 M Tris (hydroxymethyl) aminomethane and 0 1 N HCl ("Tris HCl")

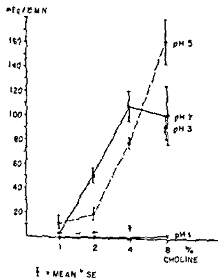


Fig 1

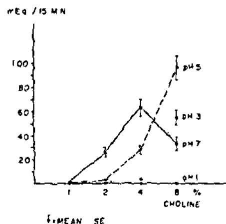


Fig 2

Fig 1 Acid output ($\bar{x} \pm S\bar{x}$) to antral administration with graded concentrations of choline at different pH levels in three Pavlov puoch dogs (264 337 337) Each point is the mean acid output for the last two 15 min periods at each dose

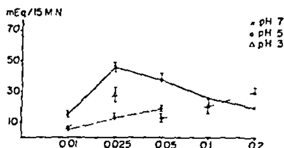
Fig 2 Acid output ($\bar{x} \pm S\bar{x}$) to antral administration with graded concentrations of choline at different pH levels in two Heidenhain pouch dogs (164 237) Each point is the mean acid output for the last two 15 min periods at each dose

TABLE I Influence of acidity on stimulatory potency on gastric acid secretion of 4 % choline solutions instilled in isolated antral pouches of 2 Heidenhain pouch dogs (164 212) and one Pavlov dog (264) pouch Stimulatory effect at pH 7 = 100 %

Antral pH	Expts	DOG 164 Mean acid output mEq/4 h	%	Expts	DOG 212 Mean acid output mEq/4 h	%	Expts	DOG 264 Mean acid output mEq/4 h	%
7	4	4 06	100	3	1 22	100	2	5 24	100
5	3	0 80	19 7	2	0 62	50 8	2	3 68	70 2
3	3	0 16	3 9	2	0 31	25 4	1	1 02	19 4

anxiety, restlessness relaxation of the nictating membrane salivation and defaecation In all experiments on one dog and in single experiments on other dogs these symptoms were so pronounced that further experiments with 8 % choline could not be performed The cholinergic effects which appeared indicate absorption of choline from the antrum These toxic effects may explain the decline in secretory response to 8 % solutions at pH 7

In another series of instillation experiments using 4 % choline of pH 7, 5 1 2 identical results were obtained (Table I)



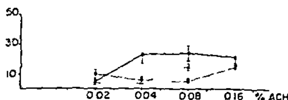


Fig 3 Acid output in a Pavlov pouch dog (264) during 2 h antral perfusion with a single dose each time of acetylcholine (top) and during administration of graded doses continuously in dose response curves (bottom) at various pH levels ($\bar{x} \pm S\bar{x}$)

TABLE II Acid output ($\bar{x} \pm S\bar{x}$) in Pavlov (P) and Heidenhain pouch (H) dogs during dose response curves with acetylcholine at various pH levels Statistical significance has been calculated between responses at pH 7 and pH 5 or pH 3 (* 0 01 < p < 0 05 ** = 0 001 < p < 0 01 *** = p < 0 001)

		Mean acid Output (mEq/15 min) in response to antral perfusion with acetylcholine (ACh)			
%	ACh	0 02	0 04	0 08	0 16
Dog	pH				
	3	0—0**	0 01—0 01***	0 01—0 03***	0 03—0 03***
164	5	0—0 03	0 03—0 04***	0 03—0 06***	0 04—0 08**
(H)	7	0 02 01	0 07 01	0 13 01	0 15 01
	1	0 01—0 02	0 01—0 01	0 01—0 01	0—0 01
	3	0 04 03	0 04 01**	0 15 ± 02	0 16 06
264	5	0 10 03	0 06 02*	0 06 ± 01**	0 14 ± 03
(P)	7	0 05 02	0 23 06	0 24 ± 05	0 21 ± 04
%	ACh	0 10	0 20	0 40	0 80
Dog	pH				
	1	0—0	0—0 01	0 01—0 01	0—0 02
	3	0 05 ± 01	0 06 02	0 07 ± 02	0 08 ± 02
237	5	0 06 ± 01	0 10 01	0 11 01	0 13 ± 01
(H)	7	0 05 ± 02	0 05 01	0 10 01	0 10 ± 02
%	ACh	0 005	0 01	0 02	0 04
Dog	pH				
337	3	0 06	0 05	0 13	0 29
(P)	7	0 20 ± 05	0 56 11	0 57 ± 04	0 53 ± 05

TABLE III

Glycine conc		0 2 M	0 4 M	0 8 M	1 6 M	3 2 M
Dog						
335	pH 7	0 00 ± 0	0 09 ± 04	0 18 ± 06	0 31 ± 04	0 32 ± 05
	5		0 00 ± 0	0 06 ± 04	0 16 ± 09	0 20 ± 11
	3		0 04 ± 03	0 10 ± 04	0 13 ± 03***	0 16 ± 05*
337	pH 7		0 34 ± 09	0 70 ± 09	0 74 ± 09	0 97 ± 05
	3		0 13 ± 10*	0 24 ± 01***	0 50 ± 0 00*	0 48 ± 12**
415	pH 7		0 14 ± 04	0 49 ± 16	0 57 ± 08	
	5	0 00 ± 0	0 05 ± 03	0 22 ± 10	0 37 ± 05*	
	3		0 09 ± 02	0 21 ± 08	0 25 ± 11*	
511	pH 7		0 03 ± 01	0 24 ± 03	0 85 ± 10	0 74 ± 13
	5		0 04 ± 04	0 15 ± 02*	0 12 ± 01***	0 09 ± 02**
	3		0 01 ± 0	0 03 ± 01***	0 05 ± 01***	0 01 ± 01***
656	pH 7	0 18 ± 06	0 37 ± 06	0 46 ± 07	0 46 ± 06	
	5	0 34 ± 19	0 20 ± 10	0 31 ± 12	0 46 ± 06	
	3	0 12 ± 02	0 13 ± 05**	0 40 ± 11	0 66 ± 06	

* = 0 01 < p < 0 05
** 0 001 < p < 0 01
*** p < 0 001

Antral irrigation with acetylcholine

In 2 Pavlov dogs and two Heidenhain dogs acetylcholine was administered in the antral pouch In both instillation and perfusion experiments it was difficult to demonstrate a true dose response relationship (Fig 3 and Table II) From Table II is also seen that the sensitivity to acetylcholine as a stimulus varied greatly among the dogs Despite this gastrin release in response to acetylcholine showed a clear pH dependency Responses to antral irrigation with acetylcholine at pH 5 were mostly considerably lower than those to acetylcholine at pH 7 in some instances even statistically significant (Table II) At pH 3 the responses were regularly further reduced

Antral irrigation with glycine

Perfusion of antrum in 5 Pavlov pouch dogs with graded dose of glycine 0 2—3 2 M solutions) at pH 7 produced graded acid responses from the fundic pouches (Table III) Solutions of glycine of pH 5 gave lower acid responses at all dose levels in 3 of 4 dogs Differences of secretory output varied among doses with regard to level of statistical significance A further reduction of acid output was obtained if glycine solutions were acidified to pH 3 in some dogs (Table III) Fig 4 summarizes results in all dogs in which glycine was administered at pH 7 5 and 3 Similar inhibitory effects of acid on the stimulatory potency of glycine were obtained in 4 Heidenhain pouch dogs during 3 h instillation of 0 3 0 4 0 8 and 1 3 M glycine solutions buffered to pH 7 5 and 3

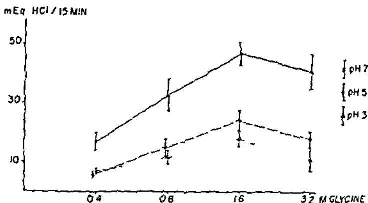


Fig 4 Dose response curves to graded doses of glycine at pH 7, 5 and 3 from 4 Pavlov pouch dogs (335, 415, 511 656) Each point indicates mean acid output for the last two 15 min periods at each dose level and vertical bars standard error of the mean.

Antral irrigation with ethanol

In two Pavlov and three Heidenhain pouch dogs the antral pouch was perfused with graded doses of ethanol Fig 5 shows dose-response curves with 4 to 32 % ethanol at pH 7 pH 3 and pH 1 in one Pavlov pouch dog No significant difference was found between secretory responses at pH 7 and pH 3 ($p > 0.05$) at any dose level ferences between secretory responses at pH 3 and pH 1 were significant ($p < 0.05$) except with 32 % ethanol A lower secretory response after 32 than after 16 % ethanol solution (Fig 5) was rather common during antral perfusion (cf Table III Elwin 1969 a) and could perhaps be explained by local toxic effects on the cells of the antrum mucosa by such high ethanol concentrations

Similar results were obtained in the 2 h perfusion experiments in the Heidenhain pouch dogs

Gastrin release by chemical stimulants compared with maximal histamine response

One way of expressing the potency of a compound as a gastric secretory stimulant is to compare its effects with the maximal response to histamine The maximal response to iv histamine was determined in 2 Heidenhain pouch and 5 Pavlov pouch dogs Table IV gives secretory responses to antral stimulation with the various chemicals used as percentage of maximal response to histamine Choline was the most potent stimulus Responses to antral perfusion with 4 % choline at pH 7 in Pavlov pouch dogs reached the same level of secretion as that of maximal histamine stimulation. At pH 5 the response to 8 % choline exceeded the maximal response to histamine in both Heidenhain and Pavlov pouch dogs Acetylcholine ethanol and glycine were less potent Acetylcholine and glycine reached a mean of 60—65 % of maximal histamine response at the most in Pavlov dogs whereas the effect of ethanol was just about 1/3 of the response to histamine However in one dog (337) the response to the highest dose of glycine applied in the antral pouch at pH 7 reached the same level as during maximal histamine stimulation (or 101 %) The results with choline

Fig 5 Dose response curves in a Pavlov pouch dog (335) for acid output ($\bar{x} \pm S\bar{x}$) in response to antral administration of ethanol at various pH levels

TABLE IV Mean acid output of Heidenhain and Pavlov pouch dogs in antral dose response experiments with different chemical stimulants expressed as percentage of maximal response to intravenous histamine (= 100 %) Figures in brackets indicate each dog's number

	Heidenhain pouch dogs				Pavlov pouch dogs			
Choline	1	2	4	8 %	1	2	4	8 %
pH 7	3	32	81	52	7	61	192	111
5	1	3	37	125	14	21	97	199
3	2	2	6	72	4	5	12	111
1	0	0	0	0	1	1	1	3
			(237	164)			(264	335, 337)
ACh	0.1	0.2	0.4	0.8 %	0.005	0.01	0.02	0.04 %
pH 7	10	10	20	20	22	63	64	60
5	12	20	21	25	7	6	15	33
3	10	12	14	16				(337)
1	0	2	2	2				
				(237)				
	0.02	0.04	0.08	0.16 %	0.02	0.04	0.08	0.16 %
pH 7	2	6	12	14	6	30	31	27
5	1	2	4	5	13	8	8	18
3	0	1	2	3	0	3	21	27
				(164)				(264
EtOH	4	8	16	32 %	4	8	16	32 %
pH 7	12	29	10	—	12	22	24	19
				(327)				
3	4	18	18	18	8	26	35	36
				(164				
1					1	6	8	14
								(335)
Glycine					0.4	0.8	1.6	3.2 M
pH 7					22	41	50	64
5					9	24	37	19
3					8	22	38	29
							(335	337, 415, 656)

and glycine clearly demonstrate that the release of endogenous gastrin by chemicals applied to the antral mucosa can produce the same secretory level from a Pavlov pouch as maximal histamine stimulation.

Discussion

The major feature of this study is the pronounced difference in pH dependency between gastrin release in response to ethanol and the other compounds. In general inhibition of responses to antral irrigation with choline, acetylcholine and glycine occurred already at a moderate reduction of pH, in most instances at pH 5 whereas pH of the antral content had to be reduced to pH 1 in order to achieve inhibition of responses to ethanol (*cf* also Elwin 1969 a). These findings may indicate different modes of inhibitory action of acid. The fact that high acidity of the antral content effectively blocks vagal release of gastrin (Andersson and Olbe 1964) suggests that one mode of action of acid may be interference with the mechanism of gastrin release. Whether this is due to a direct inhibitory effect of acid on the gastrin releasing cells *or that inhibitory impulses are transmitted by way of pH sensitive receptors at the* mucosal surface with connections to the gastrin cells is not yet known. It is of interest to note that the pH sensitivity of antral release of gastrin to ethanol is almost identical with that to vagal release of gastrin (Elwin 1969 a). This may indicate the same mechanism of acid inhibition of gastrin release by ethanol and vagal release of gastrin.

How then could the relatively high pH sensitivity of gastrin release to choline, acetylcholine and glycine be explained? A major difference can be found in physicochemical properties between ethanol and the three other compounds. Ethanol is *unionized at all pH levels. Choline and acetylcholine should according to their pK* values be nearly completely ionized at all pH levels studied. As to glycine with its amphoteric characteristics it appears as an acid below and as a base above its isoelectric point (pH 6.0). Therefore possibilities exist that the inhibitory effect of *pH variations on gastrin release by glycine is due to* changes of ionization of the glycine molecule. At pH levels below pH 5—6 the part of the molecule that possesses gastrin releasing property could thus have been more or less inactivated.

On the other hand, both choline and acetylcholine are chemically strong bases which consequently should be completely ionized at all pH levels below 7—8. Therefore the proposed mechanism of acid inhibition discussed above is very unlikely in explaining the rather high pH sensitivity of gastrin release with these 2 agents. We have at present no clear evidence of how acid interferes with the gastrin releasing action of choline and acetylcholine. However, with high concentrations of choline (4—8 %) at pH 7 within the antral pouch all dogs showed pronounced symptoms of cholinergic excitation indicating absorption of choline through the antral mucosal membrane. Analysis of the venous blood has shown an increase of blood concentration of choline with 200—300 per cent (Andersson and Elwin 1968). On the other hand, no dogs showed cholinergic side effects when *pH of choline solutions* had been reduced to pH 5 and below, and determinations of choline in blood during

irrigation of antrum with acidified choline solutions showed no change in blood choline levels Therefore absorption of choline through the antral mucosa shows a clearcut pH-dependency The penetration of choline to a receptor site or to the gastrin cell might thus be blocked by acid Similar physico chemical characteristics of choline and acetylcholine may be suggestive for a similar mechanism of acid inhibition of gastrin release to acetylcholine

Gastric acid responses to antral irrigation with choline are of interest in other respects too We found choline to be the most potent of all gastrin releasing substances tested Of particular interest is the finding that Heidenhain pouch responses to antral stimulation with choline reached up to and even above the maximal response to histamine From several studies (Passaro and Grossman 1964, Andersson and Grossman 1965) it is known that the maximal response of Heidenhain pouches to exogenous gastrin is at the most only 50 per cent of their maximal response to histamine We interpret the high response to choline in Heidenhain pouches as being due to a combined action of released gastrin and cholinergic sensitization of parietal cells by the absorbed choline

Acetylcholine has been characterized as the most protent gastrin releasing agent (Cooke and Grossman 1968) It was surprising to find that acetylcholine did not produce such high levels of secretion as did choline Sensitization of parietal cells by absorbed acetylcholine is most unlikely to occur because of its rapid inactivation in the blood and in the liver Possibly, local application of highly concentrated solutions of ACh might interfere with blood flow through the antral mucosa

The present results indicate a complex inhibitory mechanism of acid on release of gastrin At very low pH levels (pH 1—2) acid may produce a general intensitivity of the gastrin releasing mechanism to all stimuli — this was observed when ethanol was used as a gastrin releasing agent (*cf* also acid inhibition of vagal release of gastrin Andersson and Olbe 1964) Other modes of action of acid most likely include effects secondary to changes of ionization and absorption of the stimuli

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From the Department of Zoophysiology, University of Göteborg, Sweden

Adrenergic Innervation and Drug Responses of the Oval Sphincter in the Swimbladder of the Cod (Gadus morhua)[1]

By

STEFAN NILSSON

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Abstract

NILSSON, S. *Adrenergic innervation and drug responses of the oval sphincter in the swimbladder of the cod (Gadus morhua).* Acta physiol. scand. 1971. 83. 446—453.

The effects of drugs and of electrical stimulation of nerves on the circular and radial smooth muscles of the oval sphincter in the cod swimbladder have been studied. Opening of the oval seems to be caused by liberation of noradrenaline from vagal fibres in the muscularis mucosae. *The noradrenaline relaxes the circular muscles by a beta effect and contracts the radial muscles by an alpha effect.* Acetylcholine contracts both circular and radial muscles, but no definite evidence for the existence of cholinergic fibres have been obtained. Closing of the oval may occur due to lack of adrenergic impulses. Adrenergic varicose nerve fibres have been observed running in both circular and radial muscle bundles, by use of a fluorescent histochemical method.

The teleostean swimbladder is innervated by branches from the vagus nerve and the splanchnic nerve (Nicol 1952, Fänge 1953, 1966). Fahlén *et al.* (1965) demonstrated by fluorescent histochemistry adrenergic innervation of the muscularis mucosae and of blood vessels in the gas gland of the cod. Ganglionic cells in the area of the oval in *Perca fluviatilis* has been demonstrated by Saupe (1940) and Fahlén *et al.* (1965) found such cells close to the gas gland and rete mirabile.

Different kinds of adrenergic receptors have been demonstrated in different parts of the swimbladder of the eel (Nilsson and Fänge 1967), the resorbent part having exclusively beta receptors, while the secretory part seems to have both alpha and beta receptors.

The swimbladder of the cod is divided into a secretory and a resorbent part by an oval sphincter in the dorsal part of the swimbladder. The structure and mode of function of this oval was described in another *Gadus* species (*Gadus pollachius*) by Woodland (1910—13). The oval sphinchter structurally somewhat resembles that

[1] Part of this work was presented at the Meeting of the Scandinavian Physiological Society in Stockholm. *Acta physiol. scand.* 1970. 80. 36 A—37 A.

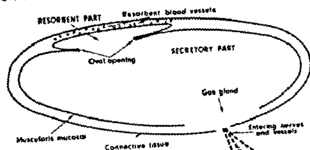


Fig 1 *Gadus morhua* Diagrammatic figure showing the main organization of the cod swimbladder

of the iris of the vertebrate eye, consisting of circular and radial smooth muscle bundles Gases are transported into the swimbladder by the gas gland with its counter-current vascular bundles, and resorption takes place by a net of blood vessels in the dorsal part of the swimbladder (Fig 1) During resorption the oval opens and facilitates contact between the gas and the resorbing blood vessels Opening of the oval can be induced by stimulation of the intestinal branches of the vagus nerve and closing is obtained when these are cut Closing of the oval is thought to occur because of activity in cholinergic intramural neurons while opening is thought to be induced by adrenergic fibres running in the vagus nerve (Fänge 1953

The fluorescent histochemistry was carried out according to Falck and Owman (1962) Pieces of the muscularis mucosae from different parts of the swimbladder were stretched over microscope slides and placed in a desiccator containing phosphorous pentoxide The preparations were left in the desiccator for about 2 h In a few expts the preparations were frozen in liquid propane condensed in liquid nitrogen and freeze dried in a vacuum desiccator at $-25°C$ for 2—3 days

Results

Physiological investigation in situ

Stimulation of the right or left vagus outside the swimbladder caused rapid opening of the oval followed by closure as soon as the stimulation was stopped No effects could be seen on the muscularis mucosae when the splanchnic nerve was stimulated

Movements of the oval during or after electrical stimulation were unaffected by atropine ($10^{-[illegible]}$—10^{-3} g/ml) or hexamethonium (10^{-4}—10^{-3} g/ml) present in the saline of the swimbladder cavity and bathing both the gas gland and the nerves outside the swimbladder Nicotine (10^{-4}—10^{-3} g/ml) caused opening of the oval and thus reduced these movements, and local contractions of the radial muscles could be seen if a drop of nicotine was placed on the muscularis mucosae Noradrenaline (10^{-6} g/ml) or tyramine ($10^{-[illegible]}$ g/ml) both caused opening of the oval

Experiments with isolated circular muscles in vitro

Acetylcholine (10^{-8}—$10^{-[illegible]}$ g/ml) (Fig 2 2) carbacholine (10^{-7}—$10^{-[illegible]}$ g/ml) or pilocarpine (10 g/ml) did all contract the circular muscles and these contractions were abolished by atropine (10^{-6} g/ml) (Fig 2 2) The ganglionic blocking agents nicotine hexamethonium or mecamylamine (all 10^{-4} g/ml) were without blocking effect but nicotine (5×10^{-5}—10^{-4} g/ml) relaxed the preparation (Fig 2 3) This effect of nicotine could be seen even though either hexamethonium mecamylamine or the beta receptor blocking agent propranolol was present in the bath

Isoprenaline (10^{-9}—10^{-5} g/ml) adrenaline (10^{-7}—10^{-5} g/ml) noradrenaline (10^{-7}—$10^{-[illegible]}$ g/ml (Fig 2 5 phenylephrine (10^{-6}—10^{-5} g/ml) (Fig 2 4) or tyramine (10^{-5} g/ml) all caused relaxation Relaxation produced by these drugs could be prevented by the beta receptor blocking agents sotalol or propranolol (both 10^{-6}—10^{-5} g/ml)

Electrical stimulation of intramural nerves caused relaxation of the preparation. Relaxations produced in this way could be repeated with intervals of 4 min for several hours When sotalol or propranolol (both 10^{-6} g/ml) was added to the bath the relaxations were blocked Fig 2 1) Guanethidine (10^{-5} g/ml) also blocked electrically induced relaxations within about 30 min No contractions could be seen during electrical stimulation after beta receptor blockade although frequency duration and voltage were varied from 1 100 c/s 0 2 –20 ms and 1—50 V respectively

Fluorescent histochemistry

Stretch preparations from the muscularis mucosae showed dense green fluorescence which was localized to nerve trunks and fine varicose terminals (Fig 3 2) The nerve trunks follow blood vessels from the hilus of the swimbladder to the oval edge sending off fibres to the muscularis mucosae and to some degree to blood vessels along its course as well (Fig 3 1) From the oval edge bundles of varicose fibres emerge following radial smooth muscles (Fig 3 3) A large portion of varicose fibres and nerve trunks follow circular muscles close to the oval edge and there are also fibres which run to the resorbent mucosa No other fluorescent structures like cell bodies were observed in the muscularis mucosae in the region around the oval No advantages were seen using the freeze-drying technique compared to drying the preparations at room temperature as described

Discussion

The opening of the oval following vagal stimulation was shown by Fange (1953) who also suggested an adrenergic innervation of the oval smooth muscles as responsible for this effect Although the splanchnic nerve anastomoses with the vagus it is not involved in the regulation of the oval but seems to innervate blood vessels of the gas gland (Nilsson unpublished) Opening of the oval seems to be due to two effects i e relaxation of circular muscles and contraction of radial muscles Relaxation of the circular muscles is probably a beta adrenergic effect since isoprenaline is the most potent drug to cause relaxation compared to adrenaline noradrenaline and phenylephrine and since relaxations produced by electrical stimulation of intramural nerves are blocked by beta adrenergic blocking agents

Contraction of radial muscles can be obtained with acetylcholine but since atropine in doses which block all effects of added acetylcholine fails to block contraction caused by stimulation of intramural nerves the transmitter substance most probably is not acetylcholine but probably is a catecholamine The effect of acetylcholine is direct not mediated by an adrenergic transmitter since phentolamine in concentrations which block electrically induced contractions does not block the effect of added acetylcholine This effect of acetylcholine was also observed in the eel swimbladder (Nilsson and Fange 1967) The contractions caused by adrenergic drugs appear to be alpha effects since isoprenaline is considerably less potent than adrenaline noradrenaline or phenylephrine

Euler and Fange (1961) found in the cod swimbladder that noradrenaline is present in greater amounts than adrenaline and Fahlen *et al* 1965 have stated that the fluorescence in the nerves of the swimbladder originates from noradrenaline

Nicotine could conceivably act by stimulation of postganglionic adrenergic neurons which may be represented by ganglionic cells situated in the muscularis mucosae close to the oval edge Such cells have been reported by Saupe 1940) to be present in the oval region of the swimbladder of another teleost *Perca fluviatilis*



Fig 3 *Gadus morhua* swimbladder 1 Fluorescent nerve trunk following a blood vessel from the hilus of the swimbladder to the oval edge Note the weakly fluorescent blood vessel beside the nerve trunk Calibration 100 μm 2 Fine varicose nerve terminals in the muscularis mucosae at some distance from the oval opening Calibration 100 μm 3 Varicose nerve bundles following the radial smooth muscles from the oval edge Calibration 200 m

Experiments with isolated radial muscles in vitro

Contraction of these preparations could be observed after addition of acetylcholine or carbacholine (both 10 —10 g/ml) the contraction beeing blocked by atropine (10^{-6} g/ml) (Fig 2 8 while alpha adrenergic blocking agents were without effect (Fig 2 7) Nicotine hexamethonium or mecamylamine (all 10^{-4} g/ml) seemed to lack blocking effects towards acetylcholine (Fig 2 8) but nicotine (10^{-5}—10^{-4} g/ml) caused weak contraction of the preparation

Adrenaline noradrenaline phenylephrine (all 10^{-7}—10 g/ml or tyramine (10^{-5} g/ml) caused contraction of the preparation which could be blocked by the alpha adrenergic blocking agents dihydroergotamine yohimbine phenoxybenzamine or phentolamine (all 10^{-6}– 10 g/ml Fig 2 8 Isoprenaline (10 g ml) had weak effects and did sometimes cause a slight relaxation

Electrical stimulation of intramural nerves in these preparations caused contractions which could be repeated for hours at four min intervals The contractions were blocked by phentolamine (Fig 2 7 phenoxybenzamine Fig 2 6 or yohimbine (all 10^{-6}—10^{-5} g/ml) and by dihydroergotamine (10 g/ml Guanethidine (10^{-5} g/ml) abolished the electrically induced contractions within 30—40 min

Fluorescent histochemistry

Stretch preparations from the muscularis mucosae showed dense green fluorescence which was localized to nerve trunks and fine varicose terminals (Fig 3 2) The nerve trunks follow blood vessels from the hilus of the swimbladder to the oval edge sending off fibres to the muscularis mucosae and to some degree to blood vessels along its course as well (Fig 3 1) From the oval edge bundles of varicose fibres emerge following radial smooth muscles (Fig 3 3) A large portion of varicose fibres and nerve trunks follow circular muscles close to the oval edge and there are also fibres which run to the resorbent mucosa No other fluorescent structures like cell bodies were observed in the muscularis mucosae in the region around the oval No advantages were seen using the freeze drying technique compared to drying the preparations at room temperature as described

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However, no such cells have been reported in the oval region of the cod swimbladder (Fange 1953, Fahlen *et al.* 1965). The failure of ganglionic blocking drugs to cause insensitivity to nicotine indicates that the effect of nicotine is not that of ganglionic stimulation. Propranolol does not seem to block the nicotine induced relaxation on circular strips, and therefore the relaxation is not likely to be due to liberated noradrenaline.

Numerous ganglion cells were observed by Deineka (1905) in the gas gland region in the perch (*Perca fluviatilis*). This gas gland ganglion is also present in the cod, and contains fluorescent cells (Fahlen *et al.* 1965). Euler and Fange (1961) found in denervation experiments in the cod, that the amine content in the swimbladder muscularis mucosae is not lowered by vagotomy. One explanation may be that some adrenergic fibres of the muscularis mucosae have their cell bodies in the gas gland ganglion. In the present study the vagal regulation of the oval proved insensitive to ganglionic blocking agents bathing the gas gland and the nerves outside the swimbladder. This may suggest that the ganglionic transmission is not cholinergic, and Fahlen *et al.* (1965) described fluorescent varicose terminals in synaptic contact with the fluorescent ganglion cells of the gas gland ganglion. These preganglionic fibres are thought to run in the splanchnic nerve from the coeliac ganglion and may therefore not be involved in regulation of the oval. On the other hand some of the preganglionic fibres may be vagal, anastomosing with the splanchnic nerve. The role of the gas gland ganglion therefore needs further analysis. Closing of the oval occurs when the fish is killed or when the vagal innervation is cut due to contraction of circular muscles and/or relaxation of radial muscles.

Contraction of the circular muscles can be obtained with acetylcholine, this effect being blocked by atropine, and Augustinsson and Fange (1951) reported specific cholinesterase to be present in the muscularis mucosae. However no influence on the movements of the oval during or after vagal stimulation can be seen after the addition of atropine or hexamethonium. This may lead to the conclusion that contraction of the circular muscles *in vivo* is not initiated by cholinergic nerves. It should also be noted that no contraction of the circular muscles has been seen during electrical stimulation of intramural nerves after treatment with sotalol or propranolol.

Relaxation of radial muscles has not been seen with any of the tested drugs except occasionally with isoprenaline.

It can thus be concluded that the oval sphincter of the cod swimbladder is innervated solely by adrenergic fibres, causing opening of the oval by relaxing the circular and contracting the radial muscles. The adrenergic fibres may run in the vagus nerve or, which cannot be excluded, originate from cell bodies in the gas gland ganglion which may be innervated by preganglionic fibres from the vagi. Closing of the oval may occur by lack of "opening impulses" rather than an active "closing innervation", although such an innervation cannot be completely excluded. The state of the oval when closed may be the resting state of the muscles involved, and the existence of elastic elements in the circular muscles and other mechanical properties of the muscularis mucosae are now being investigated in this laboratory.

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Acta physiol. scand. 1971. 83 454—462

From the Brain Research Laboratory, E-blocket, the University Hospital of Lund, Sweden

The Effect of Arterial Hypoxemia upon Acid-Base Parameters in Arterial Blood and Cisternal Cerebrospinal Fluid of the Rat

By

V MacMillan[1] and B K Siesjo

Received 10 May 1971

Abstract

MacMillan, V and B K Siesjo, *The effect of arterial hypoxemia upon acid base parameters in arterial blood and cisternal cerebrospinal fluid of the rat* Acta physiol scand 1971 *83* 454—462

he effects of hypoxemia on the acid base parameters of the arterial blood and CSF were studied anesthetized constant ventilated rats The results showed that there was a one to one relationship ween the increase in the CSF lactate and the decrease in the CSF bicarbonate concentration over a wide range of arterial P_{O_2} values However, the decrease in the CSF pH at constant ventilation was much less than could be expected from the decrease in bicarbonate This was due to a concomitant fall in the CSF CO_2 tension, partly caused by a fall in the arterial CO_2 tension and partly by a decrease in the P_{CO} difference between CSF and arterial blood

One of the most common causes of a cerebrospinal fluid (CSF) acidosis is brain hypoxia The acidosis is probably caused by anaerobic production by brain tissue of lactic acid which diffuses out into the CSF, and lowers the bicarbonate (HCO_3^-) concentration (Severinghaus *et al* 1963, Kjallquist *et al* 1969) There is, however, a controversy about the stoichiometrical relationship between the increase in CSF lactate concentration and the decrease in the HCO_3^- concentration In acute hypoxia of a few minutes duration a 1 1 relationship has been reported (Kaasik *et al* 1970) but in sustained hypoxia the decrease in the CSF HCO_3^- concentration does not seem to be accompanied by a corresponding increase in lactate (Mines and Sorensen 1971)

The present experiments were undertaken to study the arterial and CSF acid base changes accompanying a lowering of the arterial oxygen tension (P_{O_2}) to levels as low as 15 mm Hg for 30 min The acid base changes were evaluated by measuring the carbon dioxide tensions (P_{CO_2}) in arterial blood and in CSF at various arterial P_{O_2}, as well as the corresponding HCO_3^- and lactate concentrations The results

[1] R S McLaughlin fellow on leave of absence from the Department of Medicine (New Fo Toronto General Hospital, Toronto Canada

permitted an evaluation of the relationship between the increase in the CSF lactate concentration and the decrease in the HCO_3^- concentration The results showed that there was a 1 1 relationship between the increase in the lactate and the decrease in the bicarbonate concentration However, the decrease in the CSF pH at constant ventilation was much less than could be expected from the decrease in bicarbonate This was due to a concomitant fall in the CSF CO_2 tension partly caused by a fall in the arterial CO_2 tension and partly by a decrease in the P_{CO_2} difference between CSF and arterial blood

Methods

The experimental procedures were similar to those described in a previous publication from the laboratory (Siesjo and Nilsson 1971) therefore only the general outline of the procedures will be described

Operative and sampling techniques The experiments were pe[illegible]
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TABLE I Arterial oxygen tensions, mean arterial blood pressure (MABP), arterial carbon dioxide tensions arterial pH and whole blood lactate (mm [illegible]
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± [illegible]

P_{O_2} (range)	P_{O_2} (mean)	MABP	P_{CO_2}	pH	La	La/Py
> 100	120	150	37 8	7 396	3 28	14 1
(11)	± 4	±3	±0 7	±0 010	±0 18	±0 7
80—60	67 1	155	38 9	7 399	3 08	14 3
(11)	±1 9	±4	±0 8	±0 008	±0 24	±0 8
60—45	51 7	150	37 6	7 371	4 01	21 2
(10)	±1 3	±7	±0 8	±0 018	±0 37	±3 6
45—35	38 2	135	38 5	7 328	6 89	31 1
(9)	±1 0	±5	±0 9	±0 011	±0 54	±3 2
35—30	32 2	135	39 0	7 273	8 78	36 4
(11)	±0 3	±4	±0 7	±0 023	±1 26	±4 7
30—25	28 1	130	34 1	7 209	13 24	52 3
(8)	±0 7	±3	±1 3	±0 027	±1 24	±6 6
25—20	22 5	135	34 6	7 209	15 56	56 2
(9)	±0 5	±4	±1 4	±0 025	±0 84	±4 7
< 20	17 6	125	29 3	7 133	13 54	71 0
(5)	±0 8	±4	±1 7	±0 017	±1 24	±11 9

Results

In the material presented, attempts were made to minimize variations in factors other than oxygen tension In all animals the body temperature varied within the extreme limits 35 5 C and 38° C, but the majority had temperatures between 36 5° C and 37 5° C The arterial hemoglobin content was 12 0 g/100 ml or higher Every animal had a mean arterial blood pressure of 120 mm Hg or higher Below arterial P_{O_2} of 20 mm Hg the blood pressure almost invariably fell towards the end of the 30 min hypoxemic exposure To obtain animals with blood pressures above 120 mm Hg in this P_{O_2} range, the exposure to hypoxemia had to be reduced to 15 min Previous data (Siesjo and Nilsson 1971) dealing with arterial hypoxemia was comparable in procedure and in results and are therefore included in this study

Arterial blood Table I gives the mean arterial blood pressure, CO_2 tension, pH lactate content and lactate pyruvate ratio of animals subdivided according to arbitrary P_{O_2} ranges The table shows that a progressive nonrespiratory acidosis developed when the arterial P_{O_2} was lowered below 45—50 mm Hg There was a progressive increase in the whole blood lactate concentration and in the lactate/pyruvate ratio when the arterial P_{O_2} was reduced below about 50 mm Hg A 1 1 ratio between the increase in lactate and the decrease in base excess is evident in Fig 1

At arterial O_2 tensions below 30 mm Hg a significant fall in arterial CO_2 tension occurred, in spite of the constant ventilation Fig 2 shows the measured decrease

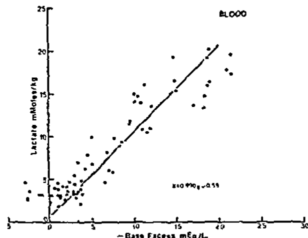


Fig 1 The relation between the whole blood lactate concentration and the calculated base excess values in rats exposed to various arterial P_{O_2} values for 30 min The regression equation is [—Base Excess] = 0 990 [blood lactate] — 0 59 The correlation coefficient (r) = 0 903 and p > 0 001

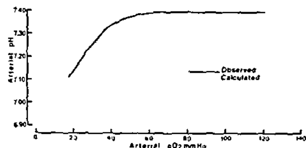


Fig 2 The relation between the arterial P_{O_2} and the decrease in the arterial pH in rats exposed to various arterial P_{O_2} values for 30 min. The curves were drawn as best fits to the individual values The calculated curve was derived from values obtained on the assumption of an unchanged arterial P_{CO_2} and a constant extracellular fluid base excess value (see text)

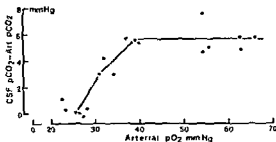


Fig 3 The relation between the arterial P_{O_2} and the measured differences between cisternal cerebrospinal fluid P_{CO_2} and arterial P_{CO_2} The cerebrospinal fluid and arterial blood P_{CO_2}'s were measured after 30 min exposure to the indicated arterial P_{O_2}'s

in arterial pH at decreasing arterial P_{O_2} Also shown is the calcuated arterial pH curve that is obtained on the assumption of an unchanged arterial CO_2 tension The pH values expected for an unchanged CO_2 tension were calculated from the Siggaard Andersen nomogram, assuming a constant extracellular fluid base excess value (5 g hemoglobin/100 ml of blood, see Siggard-Andersen 1963)

Cisternal CSF Fig 3 shows the CO_2 tension difference between CSF and arterial blood at various levels of arterial hypoxemia At P_{O_2} of 35—40 mm Hg a decrease in the difference occurred, and at P_{O_2} in the 25 mm Hg range the CSF CO_2 tension approached that of arterial blood

TABLE II Bicarbonate concentration (mmol/kg) and pH of cisternal cerebrospinal fluid in anesthetized rats maintained at various arterial P_{O_2} values for 30 min, except group P_{O_2} <20 mm Hg where the hypoxemic period was 15 min Values given are means ± S E Number of experiments within parentheses (Values for > 100 group are from control series of Kaasik *et al* (1970))

P_{O_2} (range)	P_{O_2} (mean)	CSF(HCO_3^-)	CSF pH
> 100	124 ±4	27 8 ±0 3 (4)	7 434
80—60	68 3 ±3 8	27 4 ±1 2 (4)	7 417 ±0 020
60—45	51 9 ±1 7	26 1 ±0 3 (7)	7 422 ±0 006
45—35	38 8 ±1 6	25 4 ±0 8 (5)	7 403 ±0 010
35—30	32 5 ±0 3	24 4 ±0 9 (6)	7 403 ±0 023
30—25	28 1 ±1 1	20 7 ±0 8 (4)	7 400 ±0 025
25—20	21 5 ±0 3	17 2 ±1 2 (4)	7 364 ±0 036
<20	17 6 ±0 8	19 5 ±1 1 (5)	7 448 ±0 006

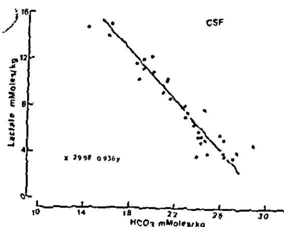


Fig 4 The relation between the cisternal cerebrospinal fluid lactate and bicarbonate concentrations measured on the same sample in rats exposed to various arterial P_{O_2} values for 30 min The regression equation is $[HCO_3^-] = 29.98 - 0.936$ [Lactate] The correlation coefficient (r) -0.914 and $p > 0.001$

Table II gives the mean CSF HCO_3^- concentration and pH obtained at the various levels of hypoxemia, while Fig 4 relates the CSF HCO_3^- and lactate concentrations in those experiments where both parameters were measured on the same sample A 1 1 relationship between increasing lactate concentration and decreasing HCO_3^- concentration is apparent An equally good correlation is obtained by taking all of the CSF lactate values measured in this and the previous paper (Siesjö and Nilsson 1971) and the CSF HCO_3^- values measured in the present study, and plotting them against the P_{O_2} (Fig 5

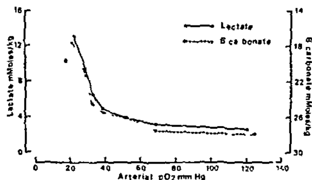


Fig 5 The relation between the arterial P_{O_2} and the cisternal cerebrospinal fluid lactate and bicarbonate concentrations in rats exposed to various arterial P_{O_2} values for 30 min The lactate values represent the means of the combined data from the present study and that of Siesjo and Nilsson (1971), while the bicarbonate values are the means of the data from the present study Values are means of P_{O_2} grouping as given in Table II The points to the left represent the mean values of the P_{O_2} < 20 mm Hg group on which the exposure was 15 min

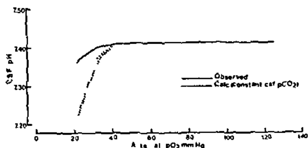


Fig 6 The relation between the arterial P_{O_2} and the cisternal cerebrospinal fluid pH in rats exposed to various arterial P_{O_2} values for 30 min Observed curve (unbroken line) derived from measured CSF CO_2 content and the CSF P_{CO_2} (derived from the measured arterial P_{CO_2} and the CSF arterial P_{CO_2} relation shown in Fig 3) Curve (open circles) derived from measured CSF CO_2 content assuming constant arterial P_{CO_2} and constant CSF arterial P_{CO_2} relationship Curve (broken lines) derived from measured CSF CO_2 content and observed arterial P_{CO_2} but with CSF arterial P_{CO_2} relationship constant (see text)

Fig 6, which relates the CSF pH to the arterial O_2 tension, shows the relatively small fall in the CSF pH even at P_{O_2} close to 20 mm Hg (unbroken line) In order to show the 'buffering' effect of the P_{CO_2} changes two theoretical curves were calcualted and compared to the experimental curve (unbroken line) One of these curves (open circles) shows the pH values which would have been obtained at the existing HCO_3^- concentrations if the CSF CO_2 tension had remained constant The difference between this line and the experimental curve thus expresses the 'regulation' of pH caused by the fall in the CSF CO_2 tension The second theoretical curve (broken line) shows the pH which would have been obtained if the arterial P_{CO_2} had changed as it did, but the CSF arterial CO_2 tension difference had remained constant The difference between this curve and the experimental curve therefore expresses the pH "regulation" occurring due to the fall of the CSF arterial P_{CO_2} difference The figure thus illustrates that the relative stability of the CSF pH in spite of a relatively marked fall in the HCO_3 concentration was due to a concomitant fall in the CSF CO_2 tension

Discussion

Despite the widespread interest in the physiological effects of hypoxemia, there has been little effort to define the effects of hypoxemia per se on the adjustments of acid-

base equilibrium of the blood or cerebrospinal fluid In most studies designed to produce an acute or chronic reduction in arterial oxygen tension, an accompanying respiratory alkalosis has served to complicate the evaluation of the specific role of oxygen lack (see Severinghaus *et al* 1963) In others, where ventilation was controlled or allowed to compensate for hypoxemia, carbon dioxide was added to the gas mixtures or inspired air in order to maintain a constant end tidal P_{CO_2} (Lundsgaard and Hansen 1966, Sapir *et al* 1967), thus adding an exogenous factor to the evaluation of the acid base changes observed In this study we have recorded the acute acid base responses of the rat, over a wide range of arterial oxygen tensions while maintaining ventilation constant, and allowing the arterial carbon dioxide tensions to reach those levels dictated by the alterations in oxidative metabolism produced by the hypoxemic exposure

As previously observed in this model (Siesjo and Nilsson 1971) a progressive non respiratory acidosis developed once the arterial oxygen tension was reduced below 50 mm Hg, with a mole to mole relationship between the measured increase in blood lactate and the decrease in base excess The arterial pH showed the expected decline with the progressive accumulation of lactic acid, however, the pH decline was partially counteracted by the fall in arterial carbon dioxide tension that occurred once the arterial oxygen tension was reduced to below 30 mm Hg The magnitude of the buffering effect of the decreased arterial carbon dioxide tension is shown in Fig 2 As alveolar ventilation was maintained constant, the decrease in arterial carbon dioxide tension observed with severe hypoxemia most probably represents a decrease in total body carbon dioxide production secondary to hypoxic suppression of oxidative decarboxylations

As the cerebrospinal fluid is a bicarbonate-containing fluid with a low protein content, it has a low buffer capacity to acute changes in carbon dioxide tension (see Fencl 1971) Thus changes in carbon dioxide tension will lead to relatively large changes in pH with no measureable changes in the HCO_3^- concentration Because of these facts, we felt it necessary to determine the effect of hypoxemia on the arterial -CSF carbon dioxide tension interrelationships In the normal rat there is a 5 5 to 6 5 mm Hg difference between CSF and arterial carbon dioxide tension (Brzezinski *et al* 1967) Our results indicate that once the arterial oxygen tension reaches 40 mm Hg or lower a rapid decline in the CSF-arterial carbon dioxide tension difference occurs At arterial oxygen tensions of about 25 mm Hg the CSF P_{CO_2} approximates that of the arterial blood The most probable explanation is that this is secondary to the marked increase in cerebral blood flow that occurs at this level of hypoxemia (Kogure *et al* 1970) A second factor to be considered is a reduced production of carbon dioxide by the cerebral tissues but this probably only occurs at very low oxygen tensions (Kety and Schmidt 1948 Shimojyo *et al* 1968) Preliminary observations of simultaneous measurements of arterial, cerebral venous, and CSF P_{CO_2} at arterial P_{O_2} of 25—30 mm Hg reveal that the CSF P_{CO_2} approximates the arterial value, while a difference of 3 4 mm Hg or so is maintained between venous and arterial values Thus in this situation the CSF P_{CO_2} is probably an inaccurate mea-

urement of mean tissue CO_2 tension in the supratentorial parts of the brain and in the extra cellular fluids of these parts (cf Ponten and Siesjo 1966)

When the CSF pH is calculated by using the HCO_3^- concentration derived from the measured total carbon dioxide content and the CSF P_{CO_2} derived from the relationship shown in Fig 3, there is little change in the CSF pH until an arterial oxygen tension of less than 25 mm Hg is reached This is remarkable in the presence of the 6–7 mmol/kg increase in the CSF lactate content and the equally large decrease in CSF bicarbonate seen at this level of hypoxemia On analysis the CSF pH is maintained relatively constant at these low levels of oxygen tension via two mechanisms As discussed above there is a decrease in the total body metabolic production of carbon dioxide with a fall in the arterial P_{CO_2} which will be reflected in a similar fall in CSF P_{CO_2} Also due to the increase in cerebral blood flow, a reduction of the CSF-arterial P_{CO_2} difference occurs which further acts to lower the CSF P_{CO_2} The relative contributions of these two compensatory mechanisms are shown in Fig 6 These compensatory mechanisms are thus separable from those seen in the fully ventilating animal who due to hypoxemic stimulation of respiration may lower the respective carbon dioxide tensions even lower

The stoichiometrical relationship between the increase in CSF lactate and decrease in CSF bicarbonate was somewhat unexpected in view of the relatively long hypoxic period Thus, if lactate appears in the CSF as the unionized acid we should expect stoichiometry, but in prolonged hypoxia there may be independent fluxes of lactate and of HCO_3^- between CSF and plasma which will disturb the original 1 1 relationship In the experiments of Mines and Sorensen 1971) there was a larger decrease in bicarbonate than in lactate indicating that either bicarbonate or lactate had disappeared from CSF to plasma Since the plasma pH and the plasma HCO_3^- concentration were held constant there is no reason to assume that HCO_3 should be lost by passive transport from CSF to plasma and it appears more probable that lactate diffused from CSF to plasma the lactate concentration of which remained low In the present experiments the CSF and plasma bicarbonate concentrations decreased by about the same amounts and since there were marked decreases in the plasma pH we should expect an increase in the electrical potential difference (ψ) between CSF and plasma see Held et al 1964 Kjallquist 1970 Sorensen and Severinghaus 1970 This would give a large increase in the electrochemical potential difference of HCO_3 calculated according to the equation see Messeter and Siesjo 1971

$$\Delta\mu_{HCO_3} = 61\,5 \log \frac{HCO_{3\ CSF}}{HCO_{3\ pl}} - \psi$$

The fact that stoichiometry between increase in lactate and decrease in bicarbonate is retained in spite of a substantial increase in the $\Delta\mu$ suggests that flux of HCO_3 from plasma to CSF was small and that the similar increases of the lactate concentrations in CSF and plasma water prevented independent lactate fluxes

the experimental conditions had given a larger increase in lactate in the CSF

plasma water, it is conceivable that lactate might have been lost from the CSF, ar that the $\Delta HCO_3^-/\Delta$ lactate ratio had been larger than unity

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The Action of Progesterone on the Sodium Transport of Isolated Frog Skin

B

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Abstract

TOMLINSON R W S *The action of progesterone on the sodium transport of isolated frog skin* Acta physiol scand 1971 *83* 463—472

It has been shown that proges erone at concentrat ons g eater than 10^{-5} M in the med bathing the outside surface of the isolated frog sk n cau es an nh b t on of the short-c current within a fe minutes The inh b t on is due to a decreased nflux of sod um w th effect on sod um efflux or chloride nflux The nh b t on is read ly el ted when the pr gesterone is added to the outs de bathing med um as opposed t the ns de bath ng ed a hough progeste one transfer across the skin is the same n both d rect ons The ade e triphosphate concentra on of the proges erone treated sk ns was not s gn f antly d ffe e n the con rols It is suggested that progesterone interacts the l p d oes f ar rd fac ng membrane pre en ing sod um nflux

Methods

The exper ments \ ere performed on the ventral abdom nal sk n of frogs *Rana tempo ana* Tl e an mals had been stored in a cold room at 4° pr or to use

Tl e frogs vere doubled p thed and the d ssected sk n \ as rinsed n R ngers solu on Tl e sk n was mounted between t o glass cl ambers and the potent al and sl ort c cu t current *measured accord ng to the method of Uss ng and Zerahn (1951) and recorded autom t call* In all of the prel minary experiments the sk n before mount ng \ as d ded nto two l al es one half acting as a control to the test half It as found necessary to t se an all glass appara u because of the large quant t es of progesterone \ h ch are ad orbed onto the polye h lene *u b ng used as connections bet een the perspex chambers and tl e glass reservo rs (see Appendix)* The glass chambers had a volume of 18 ml and tl e R ngers solut on \ as s rred w h the a d of a magnetic st rrer The sk n area vas 5 94 cm² *Sod u n nflux and effl x* were measured s multaneously us ng ²²Na and ²⁴Na The ²²Na \ as obta ned fro n the Rad o chem cal Centre Amersham England and the ²⁴Na from the Ato n c Energy Comm on Riso Denmark

Tl e ²⁴Na was counted in the presence of ²²Na t s ng a selectron c au ogamma spectrom er Appropriate correct ons \ ere made for tl e decay of the ²⁴Na Tl e ²²Na was coun ed n tl e same instrument after four weeks had elapsed to allow for the complete decay of the ²⁴Na

The *sodium transport pool* as determ ned as described by Andersen and Zeral n (1963)

Chlor de influx vas measured us ng ³⁶Cl obta ned from the Rad ocl em cal Cent e Amersham England and neutral sed th NaHCO₃ before use 1 2 / C ³⁶Cl was added to tl e R nger s solut on bath ng the outs de of tl e sk n and after 20 m n equ l b a on samples ere vithdra n from both s des at 30 m n ntervals Sc nt llat on med um Bray 1960) was added to tl e samples l cl ere tl en counted n a Packard automat c beta spectron e er The cl lor de nflux was determ ned for two 30 m n periods before and after the add on of progesterone to tl e o ts de l atl ng med um

Th *un d rect onal flux of proge terone* through tl e sk n n each d ect on was mea ured on t o sect ons of the ame sk n us ng [7α ³H] progesterone (Rad ocl em cal Cent e Amersham England wh ch as re pur fied cl romatograpl cally b fore use In a typ cal experiment tl e sk n was bathed on l oth s des with 17 ml R nger s solut on and to ne s de was added 0 t l etl anol conta n g 6 4 C of pur f ed [7α ³H] proge tro e s ec l ac ty 13? nC / g *At set t mes samples were thdra n from both s des and added to c nt lla on m e um* (Bray 1960 and counted n a Packard automat c beta spectro neter F om the rate of appearance of tl e rad oac e progesterone n the med un batl ng tl e oppos e s face and is conce rat on n the med um on tl e s de to h cl t was added tl e a f r co-eff en K *c rected f r back d ffu n as calculated*

At tl e clo e of t vo of the e periments the Ringers solut on from l l de of the sk n were extracted chromatograpl ed and counted Tl e R nger s solut ons e e ac ed ×3 w h 5 ml benzene Tl e benzene solut ons \ ere pooled and e aporated o e ght l an nf a red la np Tl e res due as d ss l ed n 100 l benzene and appl ed to nact ated l n la er plates of K eselgel F254 E Me ck A G Darmstadt Tl e plates e le el ed in chl o form acetone 80 0 and tl e pos t on of the spots ident f ed w tl a a et lamp of 256 m Areas f l e K eselgel l 5×1 0 cm e e scraped off tl e pl e d added to coun ng pl als conta n ng 5 n l f sc n llator and counted as befo e

Tl e *adeno ne t pl o phate ATP on ent a on* as m ea red in n nd proge on treated sk ns us ng a la ge pe pex l an ber wl ch afforded ea a e l e sk n f om the exterior Tl e k n rface area 7 cm² wa l atl ed on eacl s de l 40 ml of R nge s solut on Tl e potent al a d h c t ent as recorded a de b d p o l l o gesterone was added n an l an l s l n to p od ce a f nal con e n of 4×10⁻⁸M on the outs de of he te l lf f l e k n An e al l ne of etl a ol a dded to t e ou s de bath ng flu d f tl l l lf k Af er 40 t n ncuba l R ge s sol s l atl ng tl e skin we e w l d aw qu kl a d an area f sk n of 4 0 c ² l n e l be were tongs pre io l cooled w l l d l n d x de Tl e frozen k e pped in al n n um fo l and stored at 0 wl e a g l no en sa n Tl f zen k n eces we e f men ed and l om oge ed a l e px re f l d n rogen n l B a n d m l a (Braun Germa v

Tl e result ng po der a ex a ed l w l l n l of 0 3 M per l a d n a k gla s homogen er Tl e per ll a l a t was neu ral ed w l a xtu e f po a n h drox de and Tr s tl e c en at n f wl l s determ ned l 1 a n f tl e perch r c acid so that tl e f nal pH f tl e neut a ed extract was 7 5 The ATP concentra n f t extracts wi l and wi h ut a nte r al ATP tanda d was tl en determ ned u ng tl e m e h d f Rasmu sen and Nielsen 1 68

The R ngers solut on *l ad the f ll w ng compos t on m M*

NaCl 111.2 KCl 2 0 CaCl₂ 1 0 N HCO₃ 2 38 trep tom c n 0 5 g l t e

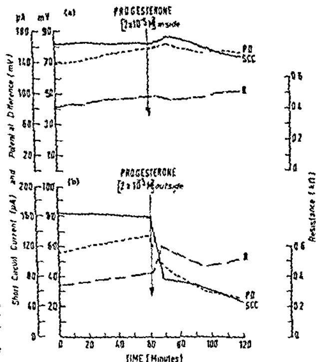


Fig 1 Changes in potential, short circuit current and resistance with 2×10^{-5} M progesterone in (a) the inside bathing medium and (b) the outside bathing medium of frog skin

Results

Effect of progesterone on the short circuit current, potential and resistance

The addition of progesterone to the Ringer's solution bathing the inside surface of the frog skin to produce a concentration of 2×10^{-5} M resulted in a small transient increase in the short-circuit current (SCC) followed by a gradual decline (Fig 1 (a)) This was accompanied by a small transient increase in the potential difference (PD) and a small transient decrease in the electrical resistance (R

The same concentration of progesterone in the Ringer's solution bathing the outside surface of the frog skin produced after 1—2 min a marked decrease in the SCC and PD with an increased electrical resistance (Fig 1 (b))

The action of progesterone on the outside surface was only partially reversible Its removal from the skin after 20 min produced a recovery of both the SCC and PD to about half and three quarters of their initial value respectively (Fig 2)

Fig 3 shows the relationship between the percentage inhibition of the SCC after 20 min exposure and the progesterone concentration of the outside bathing fluid An inhibition of 90 % being produced at a concentration of 100 µM progesterone

Effect of progesterone on sodium transport To determine whether the decrease in the SCC was caused by an increased efflux or a decreased influx double labelling experiments using ^{24}Na and ^{22}Na were employed The results depicted in Table I, showed that the inhibition was due to a decreased active transport of sodium

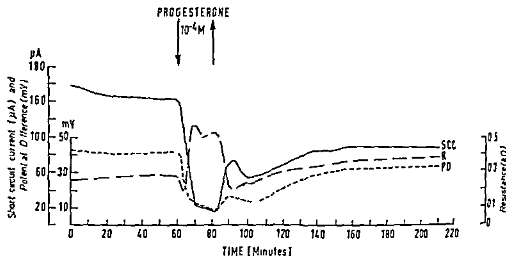


Fig 2 Changes in potential, short circuit current and resistance with the addition and removal of 10^{-4} M progesterone to the outside bathing medium of frog skin

Sodium pool determinations showed no great change in the size of the sodium pool with 3×10^{-5} M progesterone on either the inside or the outside There was however, an increase in the half-time of the sodium transported pool with 3×10^{-5} M progesterone in the outside bathing medium (Table II) There was fair agreement between the calculated active sodium flux and short-circuit current (Table II)

The effect of progesterone on chloride influx It was decided to see if the influx of other ions apart from sodium were affected by the presence of progesterone in the outside bathing medium It is apparent from Table III that the influx of chloride was relatively unaffected in the periods following the addition of progesterone to a final concentration of 2×10^{-5} M to the outside bathing medium as compared to the control periods

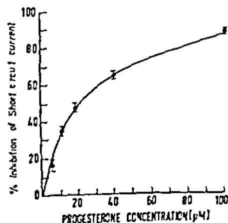


Fig 3 Mean ($\pm$SE) percentage inhibition of the short-circuit current with increasing concentrations of progesterone in the outside bathing medium

TABLE I *Sodium fluxes and short-circuit current in the absence and presence of 2×10^{-5} M progesterone in the outside medium*

Exp no	Control period				Progesterone period			
	Influx µeq/h/cm²	Efflux	ΔNa	scc	Influx	Efflux	ΔNa	scc
1	1.041	0.111	0.930	0.940	0.688	0.117	0.571	0.460
2	0.830	0.045	0.785	0.780	0.490	0.070	0.420	0.390
3	1.850	0.074	1.776	1.640	1.090	0.052	1.038	1.000
4	1.220	0.435	0.785	0.620	0.840	0.435	0.405	0.395
5	0.860	0.026	0.834	0.760	0.370	0.030	0.340	0.395
6	1.320	0.045	1.275	1.180	0.510	0.045	0.465	0.540
Mean	1.187	0.123	1.064	0.987	0.665	0.125	0.540	0.530

TABLE II *Comparison of half times ($t_{\frac{1}{2}}$) for sodium transport pool, the sodium pool, the calculated flux and current in a control period, in the presence of 3×10^{-5} progesterone in the inside bathing medium alone and together with a similar concentration of progesterone in the outside bathing medium. Area of skin 7 cm²*

Exp No	Control period				Progesterone inside				Progesterone inside and outside			
	$t_{\frac{1}{2}}$ min	Pool µEq Na	Calculated flux µEq/h	Current µEq/h	$t_{\frac{1}{2}}$d min	Pool µEq Na	Calculated flux µEq/h	Current µEq/h	$t_{\frac{1}{2}}$d min	Pool µEq Na	Calculated flux µEq/h	Current µEq/h
1	2.8	0.40	6.0	4.5	2.3	0.41	7.4	6.1	10.5 T *	—	—	1.7
2	2.5	0.49	8.1	9.8	2.6	0.39	6.2	6.8	4.5	0.39	3.6	3.3
3	2.2	0.37	6.9	4.7	2.5	0.32	3.3	4.4	3.8	0.28	3.0	2.2
4	2.7	0.22	3.4	4.3	2.6	0.20	3.2	3.0	5.7	0.23	1.7	2.6
5	2.4	0.24	4.1	4.7	2.4	0.29	4.9	4.9	5.0	0.22	1.7	2.6
6	2.0	0.31	6.4	5.3	2.0	0.37	7.7	5.0	5.0	0.30	2.5	2.4
Mean	2.4	0.34	5.8	5.6	2.4	0.33	5.8	5.0	4.8	0.28	2.5	2.4

* Could not be resolved into two components

TABLE III *Chloride influx in the absence and presence of 3×10^{-5} M progesterone in the outside bathing medium*

Exp no	Control periods		Progesterone periods	
	µeq Cl/h/5.94 cm² (i)	ii	i	ii
1				
2	0.510	0.444	0.464	0.456
3	0.154	0.204	0.228	0.176
4	0.750	1.340	1.360	1.200
5	0.550	0.630	0.490	0.570
6	2.080	2.040	1.780	1.780
	1.160	0.940	0.700	0.750
Mean				

TABLE IX *ATP concentrations in control and progesterone-treated half skins. Progesterone concentration 4×10^{-4}M in outside bathing medium. Area of skin for ATP estimations 4.9 cm²*

Exp	Control				Progesterone-treated			
	Period min	PD % change	Scc	ATP conc $\times 10^{-4}$M	Period min	PD % change	Scc	ATP conc $\times 10^{-4}$M
1	33	−9	−2	6.5	39	−52	−70	9.1
2	41	−16	−10	7.0	31	−24	−62	4.0
3	32	−11	−26	4.8	38	−47	−6[illegible]	6.6
4	38	−11	−6	5.3	31	−50	−52	5.2
5	52	−2	−15	7.6	45	−50	−67	7.0
6	34	−35	−35	5.4	42	−57	−68	2.3
7	39	−8	−7	8.4	34	−46	−66	5.5
8	34	−5	−4	6.4	42	−17	−68	7.4
9	43	−2	−12	8.8	3[illegible]	−44	−62	6.9
10	36	0	0	8.2	40	−50	−69	6.5
Mean	38	−5	−1	6.8	38	−44	−65	6.1

The permeability of frog skin to progesterone. The diffusion permeability of the skin to tritiated progesterone is so high that during the course of the observations (30 to 240 min) the concentration of progesterone in the Ringer's solution on the side to which it had been added falls appreciably and the concentration on the opposite side increases sufficiently to give rise to a significant back flux of progesterone.

Progesterone during its passage through the skin is broken down to some extent. In several experiments the Ringer's solution was extracted and the extract was chromatographed. Inspection of the thinlayer plate with UV light revealed two spots: the prominent spot was progesterone with an Rf 0.48 and the other spot with an Rf 0.37 was probably a reduced form of progesterone. At the end of an experiment of four hours duration the Ringer's solutions were extracted, chromatographed and counted. With [7α-³H] progesterone initially added to the outside bathing medium 68 % of the recovered radioactivity was found in the progesterone spot and 27 % in the metabolite in both the outside and inside bathing media. With 7α-³H progesterone added initially to the inside bathing medium 87 % of the recovered radioactivity was found in the progesterone spot and 8 % in the metabolite for the inside bathing medium and 59 % and 43 % respectively for the outside bathing medium.

In calculating the transfer coefficient K, allowance was made for the backflux of the tritiated compounds but since the relative concentration of progesterone and its metabolite was not known throughout the course of the observation the calculated transfer coefficient represents a combined progesterone and its metabolite coefficient.

The mean (± S.E.) transfer coefficient calculated from 20 observations on 4 paired-skin experiments was 0.00[illegible] ± 0.00034 cm²/min for inside to outside and 0.00621 ± 0.00035 cm²/min for outside to inside. The difference between the means has a significance level of P = 0.0[illegible] and cannot therefore be considered as a significant difference.

The effect of progesterone on the adenosine triphosphate concentration was investigated to see if the action of progesterone on the sodium transport was mediated through a blocking action of the energy producing metabolism of the skin Twelve half skins were exposed to 4×10^{-4} M progesterone concentration in the outside bathing medium for approximately 40 min while their control halves were exposed to an equal volume of ethanol Two of the experiments were discounted because of poor recovery of the internal ATP standard and the results of the ten experiments are shown in Table IV The mean ATP concentration of the control skins was 6.8×10^{-8} M and that of the progesterone treated skins 6.1×10^{-8} M The difference in ATP concentration was not significant ($P = 0.25$) although the SCC of the progesterone treated skin half was inhibited during the period by 65 %

Discussion

The inhibition of active sodium transport by progesterone *per se* when applied to the outer bathing medium of isolated amphibian skin or bladder does not appear to have been reported before

Imamura and Sasaki (1962) were unable to demonstrate an unequivocal effect of progesterone on the isolated skin of *Rana nigromacurata* at concentrations up to 3×10^{-4} M Progesterone has been widely used by workers using the isolated toad bladder preparations in the course of their studies of steroid action on active sodium transport Sharp and Leaf (1964) found that 10^{-4} M progesterone in the serosal medium inhibited the aldosterone response but did not report any effect of the progesterone itself Similarly Crabbe (1964) showed that 3×10^{-5} M progesterone added to the serosal surface produced no significant stimulation but did reduce the stimulation produced by aldosterone

Hollowell Frazer and Gardner (1968) have found that 3×10^{-5} M progesterone failed to inhibit sodium transport by the toad bladder under their conditions of assay Porter and Edelman (1964) using a serosal medium progesterone concentration of 7×10 M found no discernible activity compared to the same concentration of aldosterone This lack of an effect on sodium transport is also apparent in isolated frog skin when the progesterone is applied to the inner bathing medium but contrasts markedly with the inhibitory effect it has when applied to the outer bathing medium (Fig 1)

Progesterone has many known effects on intermediary metabolism It accelerates the rate of hydrolysis of denosine triphosphate in fresh rat liver mitochondria in proportion to progesterone concentrations between 2×10^{-5} M and 6×10^{-4} M (Wade and Jones 1956a) and uncouples oxidative phosphorylation (Wade and Jones 1956b) Progesterone inhibits mitochondrial respiration by a direct action upon the NAD flavoprotein region of the electron transport chain 50 % inhibition being obtained with a steroid concentration of 2.7×10^{-5} M (Vallejos and Stoppani 1967)

It would seem probable that such metabolic effect of progester ne could hay

occurred in the experiments reported here on the frog skin but it would not explain the difference in action of the progesterone when applied to the inner and outer surface as its ability to penetrate the skin is almost the same no matter to which side it is applied.

Progesterone at a concentration of 1.5×10^{-4} M produces a latent increase of mitochondrial ATPase activity (Vallejos and Stoppani 1967) which may account for the small but not significant difference in the ATP concentration of control and progesterone treated skins (Table IV). It would also seem improbable that the marked effect of progesterone when applied to the outer surface was due to an inhibition of Na-K activated ATPase as Hollowell *et al.* (1968) found that 3×10^{-3} M progesterone had no affect on the Na-K activated ATPase prepared from rat and guinea pig microsomes.

Recent evidence has been provided that naturally occurring steroids may also exert their functions by an action upon lysosomes. Weissmann (1965) showed that progesterone at concentrations above 2.5×10^{-4} M release β glucuronidase and acid phosphatase activity from granules of rabbit liver. Bangham, Standish and Weissmann (1965) also demonstrated a good correlation between the lytic effect of 2×10^{-3} M steroids on the release of acid phosphatase from lysosomes and the increase in cation leakage from lecithin-cholestrol-dicetyl phosphoric acid structures. They suggested that the steroids interact solely with the lipids of the membranes and the form of interaction is reflected by either an increase or decrease in the membrane permeability to cations. This would also seem to be an explanation for the experiments reported here on the isolated frog skin. The progesterone inhibition is elicited from the outside with a decrease in sodium influx but with no effect on sodium efflux or chloride influx.

Appendix

The Adsorption of Progesterone on Polyethylene Tubing

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During the course of experiments on the isolated frog skin using [7α H^3] progesterone it was found that a large proportion of the radioactivity was being adsorbed onto the apparatus. The apparatus used was the conventional one for short circuiting isolated membranes and consisted of perspex chambers, glass reservoirs and polyethylene tubing connections. The experiments were performed in duplicate on identical pieces of apparatus except that one had longer polyethylene tubing connections. In a typical experiment the skin was bathed on both sides with 30 ml Ringer's solution and to one side was added 40 μl ethanol containing 20 μCi of purified [7α H^3] progesterone (specific activity 62 nCi/μg). Samples were withdrawn from both sides at set times and added to scintillation medium (Bray 1960) and counted in a Packard automatic beta spectrometer. The loss of radioactivity from the solutions is shown in Table I. It is apparent that there was a large loss of radioactivity from the Ringer's solution (which could not be accounted for by uptake of the skin). The loss of radioactivity was greater in the apparatus in which the Ringer's solution was exposed to the larger surface area of polyethylene tubing.

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TABLE I (Appendix) *The adsorption of [7α—H³] progesterone on the apparatus*

Time	Percentage of radioactivity remaining	
h	Apparatus I Surface area of polyethylene tubing = 69 cm²	Apparatus II Surface area of polyethylene tubing = 31 cm²
0	100	100
0 5	20	36
1	12	28
2	7	15 6
3	5 4	11 0
4	4 6	8 6
8	3 8	6 0

Subsequent [illegible] er s solution was left in contact [illegible] wing materials, polyethylene, latex [illegible] %, 90 %, 60 % and 75 % respe[illegible] s in the region of 5 %

It was apparent that the progesterone during the course of the experiment was being adsorbed onto the polyethylene tubing The loss in these particular experiments is especially great as the Ringer s sol[illegible] of [illegible] it [illegible] pr[illegible] removal of the progesterone [illegible] tubing

The a[illegible] rted with testosterone but there wa[illegible] 1965) Westphal (1955) also repo[illegible] tubes during a 5 h ultracentrifug[illegible] that the less polar the steroid the more readily it is adsorbed

Although the experience encountered here is obviously not original and is supposedly well-known to steroid biochemists, it probably needs emphasising since it may be the reason why other research workers in the past have found no effect with certain steroids on isolated membrane preparations

This work was performed during the tenure of the Carlsberg Wellcome Research Fellowship I gratefully acknowledge the financial support of the Carlsberg Foundation for equipment and Professor Ussing for providing space and equipment and to him and his staff for their hospitality

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Acta physiol. scand. 1971. 83. 473—485

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Endogenous Inhibition of the Mechanical Response of the Isolated Rat and Guinea-Pig Vas Deferens to Pre- and Postganglionic Nerve Stimulation

By

GORAN SWEDIN

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Abstract

SWEDIN, G *Endogenous inhibition of the mechanical response of the isolated rat and guinea-pig vas deferens to pre and postganglionic nerve stimulation* Acta physiol scand 1971 *83* 473—485

Repeated periods with prolonged nerve stimulation (30 s) of the isolated vas deferens tended to depress especially the initial, rapid phase of subsequent contractions

The following findings led to the conclusion that the inhibition was induced by prostaglandin (PG) like material, released from the organ on nerve stimulation 1 The inhibition was immediately abolished on washing of the organ 2 It appeared faster in an organ bath of small volume than in one of a greater 3 It was possible to transfer the substance from one bath to influence an organ in another [illegible] both phases of contraction, th[illegible] in the last part of the hypo[illegible] substance as to exogenous P[illegible] a partial or total abolishmen[illegible] of PG-like material mainly [illegible]

It is suggested that this endogenous inhibiting process might play a modulating role in the nerve induced mechanical activity of this organ also under *in vivo* conditions

In a previous communication (Swedin 1971) it was reported that the mechanical response of the isolated vas deferens preparation is divided into 2 separate phases when the usual 5 s period of stimulation is extended to 30 s (*cf* Fig 1) It was shown that moderate concentrations of adrenergic α blocking agents in the bath or reserpine pretreatment of the animal heavily depressed or abolished the second, slower phase of contraction while leaving the rapid initial 'twitch" of the organ rather uninfluenced On the other hand, very low concentrations of prostaglandin E_1 (PGE_1 1—5 ng/ml) inhibited the 'twitch" without producing any marked change of the second phase During this investigation it was observed that shifting from 5 to 30 s stimulation periods tended to increase the amplitude of the forth coming 2 or 3 'twitches" Later on however, the twitches" especially of the guinea pig preparations, were successively depressed below the original height The present study was undertaken in order to investigate the nature of this depression

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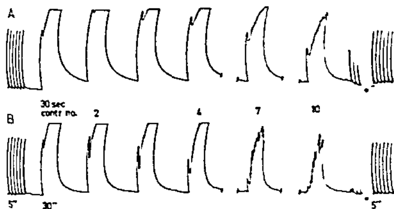


Fig 1 Isolated field stimulated (5 and 30 s) vas deferens preparations from rat (A) and guinea pig (B) in 5 ml baths 20 V, 1 5 ms duration, 5 imp/s Same amplification 1 min rest between stimulations At dots washing

in the guinea pig and the greatest degree of inhibition is seen at the lowest frequency of stimulation (2 imp/s) The responses of the *guinea pig* preparation fall into two different groups In the lower frequency range (2—5 imp s) there is only a successively increasing autoinhibition while at higher frequencies (10—25 imp s the very marked final inhibition is preceded by a definite degree of potentiation At 5 imp/s the degree of inhibition of the guinea pig preparation is at all points significantly ($p < 0.001$) greater than that of the rat as is the case with the final inhibition (contraction no 7 and 10) at 10 and 25 imp s At 2 imp s there is no certain species difference In some preparations there was a marked autoinhibition especially at low stimulation frequencies even after only one 30 s stimulation Fig 3 guinea pig vas deferens 3 imp/s) In these cases there was generally a fairly rapid spontaneous return to the original height of contraction as seen in the figure

Tests with *exogenous* NA and ACh 0 1—2 0 μg ml revealed quite unaltered responses of the vas deferens before and after the periods with 30 s stimulation

To study a possible *temperature dependence* of the described autoinhibition some experiments were performed at 25 C Fig 5 shows the same field stimulated guinea pig preparation at 37° C and 25 C After reduction of the bath temperature the degree of amplification of the Grass Polygraph had to be reduced about fourfold to give the same amplitude of the contraction registrations as that obtained with the same stimulation parameters at 37 C The 30 s contractions and relaxations were slower and very little autoinhibition of the twitches could be seen The further decrease normally seen after shifting back to 5 s stimulations was however much more pronounced and despite repeated washings only very small contractions were recorded At lower frequencies (2—4 imp/s this delayed type of autoinhibition seen at 25° C was less marked

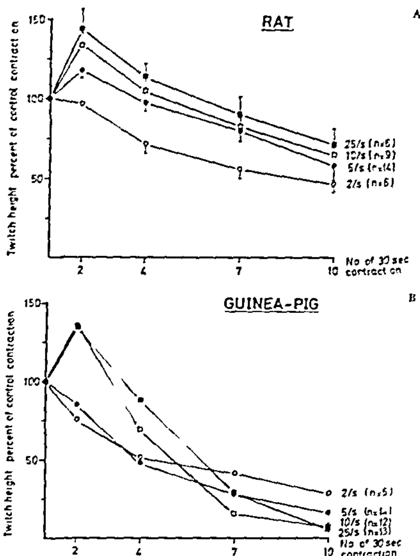


Fig 2 Influence of stimulation frequency on the degree and time course of potentiation and inhibition of the "twitches" induced by 10 subsequent 30 s stimulations in isolated field stimulated vas deferens preparations from rat (A) and guinea pig (B) cf Fig 1 A B) 5 ml baths 20 V 1 5 ms duration stimulation frequency 2 (O) 5 (●) 10 (□) and 25 (■) imp/s The "twitch" height of 30 s contractions no 2 4 7 and 10 cf Fig 1 are expressed as percent of initial control contractions Means ± s.e.m Number of expts indicated in the figures

Endogenous inhibition of the mechanical responses was seen also in preparations from rats and guinea pigs pretreated with *reserpine* (Fig 4 and 11) At 50 imp/s (Fig 11) the mechanical responses of the field stimulated guinea pig vas deferens quickly decreased to very small amplitudes even at 5 s stimulations *After washing* there was a prompt restitution of the contractions (Fig 11 B)

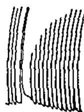


Fig 3

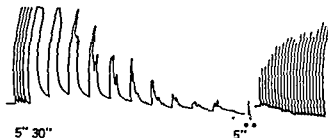


Fig 4

Fig 3 Isolated field stimulated guinea pig vas deferens, 5 ml bath 20 V, 1 5 ms duration, 3 imp/s Same paper speed at 5 and 30 s stimulations

Fig 4 Isolated field stimulated vas deferens from a guinea pig pretreated for 2 days with reserpine (3 6 and 6 mg/kg i p 48, 24 and 12 h before the expt) 1 5 ms duration, 8 imp/s 30 s interval between the 30 s stimulations Paper speed 4 times higher at 30 s stimulations at large dots washing

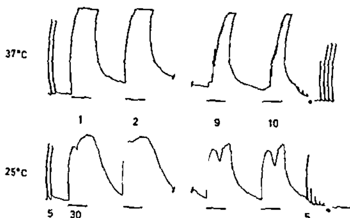
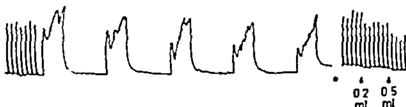


Fig 5 The same isolated field stimulated guinea pig vas deferens preparation in 5 ml bath at different temperatures 20 V 1 5 ms duration 8 imp/sec At dots washing Amplification at 25° C reduced 4 fold

In Fig 6 the effect of different *organ bath volumes* on the degree of autoinhibition is shown in two guinea pig preparations stimulated at 7 imp/s via the hypogastric nerve It is further shown that after the stimulation period Tyrode solution from the 5 ml bath has the ability to decrease the contractions of the organ in the 50 ml bath in a dose dependent way

Since this preganglionically stimulated preparation from the 5 ml bath exhibited a much more rapid and total autoinhibition than the field stimulate[illegible] organs generally do at this frequency of stimulation (*cf* Fig 2 B) the possibility of an effect on the



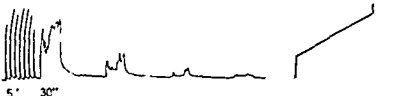


Fig 6 2 isolated guinea pig vasa deferentia from the same animal in baths of different volumes Same amplification Hypogastric nerve stimulation 25 V 1 5 ms duration 7 imp/s At dot washing After four 30 s stimulations of the organ in the small bath 0 2 and 0 5 ml of the Tyrode solution was transferred to the 50 ml bath as indicated in the figure

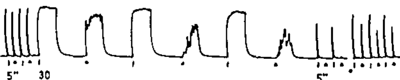
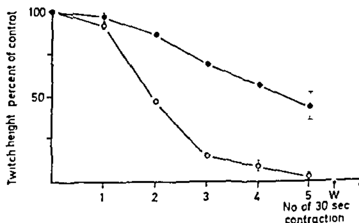


Fig 7 Isolated guinea pig vas deferens stimulated alternately preganglionically via the hypogastric nerve (▲ and postganglionically by field stimulation (|) Hypogastric nerve stimulation 25 V 1 5 ms duration 5 imp/s Field stimulation 20 V 1 5 ms 5 imp/s At dot washing



... the mechanical response of isolated guinea pig ... and postganglionically (●) at 5 imp/s in the ... Fig 7 Bath volume 5 ml At W washing

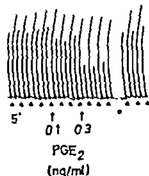


Fig 9 Effect of exogenous PGE_2 on the responses of an isolated guinea pig vas deferens stimulated alternately pre (▲) and postganglionically in a 5 ml bath The same stimulation parameters as in the exp described in Fig 7 At dot washing

ganglionic transmission was investigated in organs alternately stimulated pre- and postganglionically (Fig 7) Obviously the response to preganglionical stimulation is much more sensitive to the inhibiting agent than is the postganglionic one The different degrees of inhibition of the mechanical responses to pre and postganglionic stimulation at a stimulation frequency of 5 imp/s are summarized in Fig 8

The effects of different concentrations of *exogenous PGE_2* are shown in Fig 9 and 10 In the lowest concentrations (0 1—0 4 ng/ml Fig 9) there are definite effects only on the *preganglionically* stimulated guinea pig vas deferens while a tenfold increase in concentration is necessary to get a distinct effect on the response to *postganglionic* stimulation (Fig 10) The concentration necessary for an inhibition of the second phase of contraction falls in the range of 10—50 ng/ml (Fig 10) PGE_1 is effective on the second phase in the same concentrations

To test the possibility that PG might play a role in the autoinhibition of the vas deferens experiments were performed where ETA was added to the organ bath in concentrations of 1—20 μg/ml in order to *inhibit PG synthesis* Generally there was some direct inhibitory effect of the drug unrelated to the alkaline solvent (Fig 11 C) but sometimes a slight increase in the responses was seen immediately after the addition of the drug The long time effect of the drug was however a partial (Table I) or total (Fig 11 C) abolishment of the endogenous inhibitory mechanism

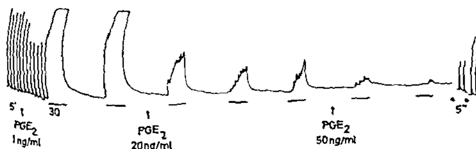


Fig 10 Effect of exogenous PGE_2 on the response of an isolated field-stimulated guinea pig vas deferens in a 5 ml bath 20 V 1 5 ms duration 5 imp/s At dot washing

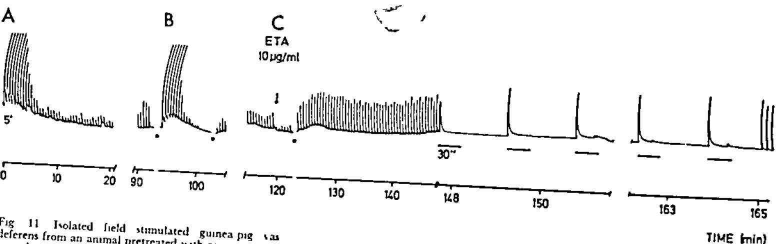


Fig 11 Isolated field stimulated guinea pig vas deferens from an animal pretreated with reserpine (see legend to Fig 5) 20 V 1 5 ms duration 50 imp/s for 5 s at 30 s intervals or 30 s at 1 min interval Of the 30 s stimulations no 1 2 3 9 and 10 are shown in the Fig 5 8 11 14 eicosatetraynoic acid (ETA) added in ammonium form At dots washing

TABLE I Effect of [illegible]

*** $p < 0.001$

	"Twitch" height (% of control) of 30 s contraction no 2	4	7	10
Control inhibition	85.4±4.9	49.7±9.0	21.8±4.9	8.2±2.3
After ETA	122.3±6.0***	103.4±5.0***	48.8±7.8**	26.0±3.7**

Finally an attempt was made to extract and characterize the inhibitory agent. After thin layer chromatography (TLC) of the lipid extracts from Tyrode solutions the fractions corresponding to PGE_1 and PGE_2 were redissolved in Tyrode solution and tested on isolated rat fundus strip preparations. From Fig. 12 it is obvious that material resembling the prostaglandins, predominantly PGE_2, was present in the stimulated Tyrode solution.

Discussion

In the present study on the isolated rat and guinea pig vas deferens it has been shown that successive periods with 30 s nerve stimulation of the organ leads to a progressive decrease of the mechanical response. At higher frequencies of stimulation (10—25 imp/s) and especially in the rat preparation this depression is preceded by enhanced contractions. The nature and degree of the endogenous inhibition is dependent on species, temperature and frequency of stimulation. The depression does not seem to be related to postjunctional fatigue of the receptors or the contractile elements of the effector cells since the responses to exogenous NA and ACh are

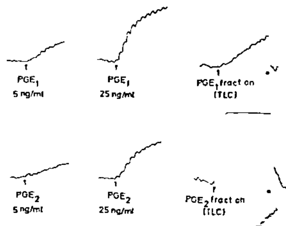


Fig. 12. Isolated strip of the fundus part of the rat ventricle in a 5 ml bath. Effects of test doses of PGE_1 and PGE_2 and 100 μl of redissolved TLC fractions (total volume 400 μl) from stimulated guinea pig vas deferens preparations (for further details see methods).

uninfluenced by periods with successive inhibition Furthermore it seems to be correlated to the liberation upon nerve stimulation of a substance which can be isolated from the bath fluid This assumption is based on the following observations 1 The inhibition is immediately abolished on washing of the organ 2 It appears faster and is more complete in an organ bath of small volume than in one of a greater 3 It is possible to transfer the substance with its inhibiting properties from one organ bath to influence an organ in another

It has been shown that PG, especially of the F series cause an effective prejunctional inhibition of the mechanical response of the isolated vas deferens to field stimulation (Euler and Hedqvist 1969 Ambache and Zar 1970 Hedqvist and Euler 1971) Furthermore PGF₂ has been shown to be released in response to nerve stimulation from the spleen of the dog (Davies Horton and Withrington 1967 1968) and cat (Gilmore Vane and Wyllie 1968)

Recently experimental data on the rabbit heart has been presented (Wennmalm and Stjarne 1971 Samuelsson and Wennmalm 1971) supporting the suggestion of an endogenous prostaglandin induced restriction of the transmitter release from sympathetic nerves (Hedqvist and Brundin 1969 Hedqvist 1970 Wennmalm and Stjarne 1971 Wennmalm 1971)

These reports were the grounds for the suspicion that PG might be involved in the reported autoinhibition of the respon es of the vas deferens to nerve stimulation Arguments in favour of this assumption are offered by the present results Firstly PGE_1 and PGE_2 were shown to inhibit not only the twitch (Euler and Hedqvist 1969) but also in higher concentrations the slower second phase of contraction which is also although to a less extent susceptible to the autoinhibition Secondly the release upon nerve stimulation of prostaglandins in the tested preparations predominantly PGE has been established Thirdly inhibition of the PG synthesis by ETA (Downing Ahern and Bachta 1970 Samuelsson and Wennmalm 1971) partly or totally abolished the endogenous inhibition induced by successive periods of 30 s stimulations That this effect of ETA really is related to a partly or total blockade of the PG synthesis and not to a direct stimulating effect on the effector cells or to direct release of neurotransmitter is indicated by the fact that the direct effect of the drug on the responses of the vas deferens to nerve stimulation rather seems to be of depressant character Furthermore studies on the isolated rabbit heart has indicated that ETA does not influence the uptake of exogenous NA (Samuelsson and Wennmalm 1971) nor does it evoke a direct release of NA from this organ (Wennmalm pers commun)

So far no reports have been presented indicating a definite effect of PG on autonomic ganglion transmission Therefore it was a rather unexpected finding that the ganglionic relay present in the last part of the hypogastric nerve (*cf* Sjostrand 1965 Ferry 1967) seemed to be sensitive and even a great deal more sensitive to the released inhibitory agent than the postganglionic neuroeffector transmission But also this fact turned out to support the hypothesis of an endogenous PG mechanism in the vas deferens as it was found that the synaptic transmission in the hypogastric

ganglion was about ten times more sensitive than the postganglionic transmission also to exogenous PG Partly this extremely high sensitivity of the hypogastric ganglion might be due to a favourable location of the nearly unprotected ganglion cells in the thin peritoneal cover at the base of the vas deferens With regard to the negative reports concerning PG effects on ganglionic transmission (Kavaalp and McIsaas 1968) a true difference in comparison to other autonomic ganglia cannot, however be excluded since this peripheral sympathetic ganglion in the hypogastric nerve also shows other divergent pharmacological properties (Bentley 1968)

Finally also the described species differences in effectiveness of the autoinhibition between the rat and guinea pig speaks in favour of a PG mechanism since the guinea pig vas deferens is more sensitive also to exogenous PG (Hedqvist and Euler 1971)

The clear decrease in degree of autoinhibition seen at lower temperature (25° C) is interesting since this might be a part of the explanation to the increased sensitivity of isolated vas deferens to nerve stimulation at lower temperatures (Della Bella Gandini and Preti 1965) There is, however, also at lower temperatures a release of the inhibiting substance, as indicated by the delayed inhibition seen after shifting back to 5 s stimulations The delay in onset of the autoinhibition might depend partly on slower diffusion of the inhibiting agent from its site of release and partly to a more longstanding effect of the released neurotransmitter

The rapid reduction especially at high frequency of stimulation of the mechanical responses of the vas deferens from reserpine pretreated animals has been repeatedly reported (Hukovic 1961, and others) This has been interpreted as a result of an exhaustion of the severely depleted NA stores in these organs From the present study (*cf* Fig 11 C) it seems however to be clear that the endogenous autoinhibition plays the major role in the reported reduction of the responses of the reserpinized vas deferens A further indication of this is the fact that the observed restoration of the nerve induced contractions of the vas deferens after incubation with NA and subsequent repeated washings of the organ (Hukovic 1961) can be reproduced with the washings only (*cf* Fig 11 B)

A comment should also be given to the reported initial potentiation of the "twitches after shifting to 30 s stimulation periods The most probable explanation for this effect is that the first 30 s stimulation will release sufficient amounts of trans mitter to cause the same kind of potentiation of the nerve induced responses of the organ as that reported for the nerve stimulated vas deferens or seminal vesicle prepa rations to low amounts of exogenous NA (Sjostrand 1961 Sjostrand and Swedin 1968 1970) The species differences as well as the different degree of endogenous potentiation or inhibition at different frequencies of stimulation might well be ex plained by different proportions of released transmitter and inhibitory substance This could be the reason why the reserpinized vas deferens is more dominated by the inhib tory agent than the control preparations From a physiological point of view it is interesting that the inhibitory mechanism is so well developed and equally ef fective in the rat and guinea pig already at very low frequencies of stimulation

imp/s) The species differences in the slope of the inhibition curves at higher stimulation frequencies might indicate differences in effectiveness of the inactivating processes of the excitatory and inhibitory agents, respectively

From the present results the conclusion can be drawn that the mechanical response of the isolated vas deferens to nerve stimulation is under a dual influence from excitatory and inhibitory agents The inhibitory agent is most probably working at a prejunctional level presumably by restricting the amount of neurotransmitter released on nerve stimulation Several experimental data has been presented supporting the hypothesis that the endogenous inhibition could be due to release of prostaglandins within the organ With regard to the effectiveness of the described autoinhibition even at very low stimulation frequencies it is tempting to suggest that this process might play a modulating role in the nerve induced mechanical activity of this organ also under *in vivo* conditions Furthermore, the existence of such an inhibiting mechanism has to be considered in further studies on the motor innervation of the vas deferens

This investigation has been supported by the Medical Faculty, Karolinska Institutet Stockholm

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Methods

The experiments have been carried out on [illegible] d g long II) The methods used for isol [illegible] in detail in earlier papers (Ottoson 1961 [illegible] and Shepherd 1969) and will [illegible] dissected free from the muscle [illegible] tied to two fine nylon rods Th[illegible] to take up slack The resting length was about 1 mm [illegible] eparations One of the nylon rods was attached through a metal lever to a loudspeaker coil (Philips AD 2300 BZ) Stretches were applied by driving the coil with electrical pulses The stimulus was monitored on the oscilloscope by means of a high sensitivity capacitance meter (Haapanen 1962) which measured the movement of the metal portion of the pulling rod

Recordings were made with calomel half-cell electrodes which were connected with the preparation through agar bridges One electrode [illegible] tact with the afferent nerve which was lifted up [illegible] coupled amplifier The Ringer solution used [illegible] KCl 2 5 mM CaCl₂ 1 8 mM, NaH₂PO₄ 0 85 [illegible] Potassium free solution had the same composition as the normal Ringer solution except that potassium was replaced by sodium The temperature of the bath was kept at +18° C

Results

Effects on spontaneous activity and spike shape

The spindle usually fires at a low irregular rate (1—2/s) when kept at resting length This activity is very sensitive to changes in the external environment of the spindle (cf Ottoson 1964, 1965) and may therefore be used as an index of the action of alterations in the ionic composition of the bathing fluid The first sign of the effect of removal of potassium was a slowly developing increase in firing rate of the resting spindle Another effect was that the spontaneous activity became very regular These changes usually appeared about 10 min after potassium had been removed from the external solution and proceeded gradually over the next 30—40 min The frequency of firing reached at the end of this period usually was 6—8 imp/s The activity then began to decrease and when the spindle had been kept in the potassium free solution for 60—90 min there was usually no sign of spontaneous activity

The changes in the spontaneous activity were accompanied by alterations in the configuration and time course of the impulses The first noticeable change was a lowering and a broadening of the action potential and the appearance of a prolonged negative afterpotential (Fig 1 records b—c) This change usually appeared at the same time as the spontaneous activity began to increase Later the impulse developed

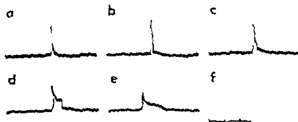


Fig 1 Changes in configuration of spontaneous action potential after removal of potassium Record a normal Ringer b 20 min c 28 min d 35 min e 40 min and f 43 min after removal of potassium from the bathing solution Time marks 25 ms

Acta physiol scand 1971, 83, 486—494

From the Department of Physiology, Royal Veterinary College, Stockholm Sweden

Impulse Activity of the Isolated Spindle in Potassium-Free Solution

By

I HUSMARK and D OTTOSON

Received 24 May 1971

Abstract

HUSMARK, I and D OTTOSON *Impulse activity of the isolated spindle in potassium free solution* Acta physiol scand 1971 *83* 486—494

The effect of removal of potassium on the impulse activity of the frog muscle spindle was studied in isolated preparations The spontaneous activity of the spindle increased over the first 30—40 min and then decreased and finally ceased These changes were accompanied by alterations in configuration and time course of the action potential characterized by a decrease in amplitude and a lengthening of the impulse which developed into a heart like action potential The sensitivity of the spindle to stretch decreased gradually with time and no conducted activity could usually be evoked by stretch 60—90 min after removal of potassium. Following return to normal Ringer the spontaneous activity and the response to stretch reappeared rapidly The observed changes can partly be attributed to the effect of potassium removal on the conducted activity of the afferent axon and partly to the effect on the transducing function of the mechano-sensitive endings

In his classical work on the frog spindle in 1931 Matthews found that the behaviour of the end organ was greatly modified by alterations in the ionic composition of the surrounding fluid These observations have later been confirmed and extended in experiments on the isolated spindle (*cf* Katz 1950, Ottoson 1964 1965) In the course of studies of the ionic effects on the adaptation of the spindle to maintained stretch it was observed that removal of potassium affected not only the transducer function of the endings (Husmark and Ottoson 1971 *b*) but also the conducted activity of the afferent fibre The present study was carried out in order to examine this latter effect more closely Such a study appeared of particular interest as recent studies of the effect of lowering the potassium concentration on the perfused giant axon (Baker Hodgkin and Meves 1964) have focused attention on the critical importance of potassium ions in the impulse generation process The present results show that if potassium is removed from the external solution the action potentials become prolonged and are changed into heart like action potentials with a plateau lasting for 40—60 ms This change is accompanied by a depression of the responsiveness of the spindle to stretch With prolonged exposure to potassium free solutions the conducted activity is abolished while the non conducted activity persists although reduced A preliminary report of these results has been published earlier (Husmark and Ottoson 1971 *a*)

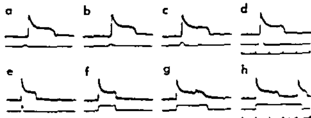


Fig 4 Effect of changes in amplitude (*a*—*d*) and duration (*e*—*h*) of stretch on heart like action potential of spindle in potassium free Ringer Records obtained 30 min after removal of potassium Time marks in *d*, 10, in *h* 20 ms

in responsiveness was accompanied by the above described changes in configuration of the impulse After the spindle had been kept in the potassium free solution for about one hour or more no impulse response could be evoked, irrespective of the amount of stretch

The response with a well developed plateau (*cf* record *d* in Fig 3) closely resembles the receptor potential obtained with a maintained stretch The behaviour of the response to alterations of the parameters of stretch clearly shows however, its all or none character Thus, the response remained unaltered by changes in the strength of a brief purely phasic stretch (Fig 4 *a*—*d*) Furthermore, increase of the duration of the stimulus did not produce any changes of the response as long as the stimulus duration did not exceed that of the plateau (Fig 4, *e*—*f*) With stretches of longer duration the initial spike remained unchanged but there appeared a second spike (records *g* and *h*) with a shorter plateau phase The duration of the plateau phase of the spikes following the initial impulse was related to the frequency of firing This could be demonstrated by using maintained stretches of different strengths as is shown in Fig 5 It can be seen that there is a gradual lengthening of the plateau of the static spikes with the adaptive decline in frequency

It has been shown in a previous study (Ottoson and Shepherd 1970) that the action potential of the spindle under certain experimental conditions can be decomposited into smaller unitary impulses representing the activity of the myelinated nerve branches within the spindle This may be explained as a breakdown of the synchronous activity and is most likely due to a prolonged refractoriness or blockage of the conducted activity in the terminal branches In the present study it was found that exposure of the spindle to potassium free Ringer caused a similar

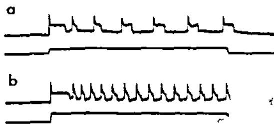


Fig 5 Change in duration of plateau phase of static spikes with frequency of firing during maintained stretch Time marks 100 ms

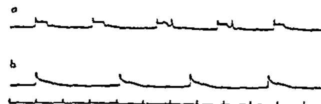


Fig. 2. Regular spontaneous activity in potassium-free Ringer. Record *a* 50 min, *b* 40 min after removal of potassium. Recordings from two different spindles. Time marks 100 ms.

into a heart-like action potential with a marked plateau (record *d* in Fig. 1). The plateau phase increased in duration and 30—40 min after removal of potassium it usually lasted for 40—60 ms (record *e* in Fig. 1). Following this stage the impulse rapidly decreased in amplitude and the decay from the plateau became still more prolonged. After soaking the spindle for about 60 min there usually remained only small wave-like potentials of long duration (record *f* in Fig. 1).

The regularity of the spontaneous discharge in potassium-free solution is illustrated in Fig. 2 which shows continuous recordings from two different spindles. It may be noticed in *a* that the heart-like action potential was sometimes followed by a second smaller impulse with a brief plateau. These spikes as well as the regular spontaneous activity may be explained as the results of a prolonged depolarization following each impulse.

Effect on response to stretch

The changes in excitability were tested by applying brief stretches at threshold for evoking a single spike. It was found that the threshold increased gradually with time after removal of potassium. The continuous change made it difficult to carry out precise threshold measurements as did also the rapid increase in spontaneous firing. Only the general trend of the alterations in the sensitivity of the spindle could therefore be determined.

The records in Fig. 3 show responses obtained with brief test stretches of approximately threshold strength before (*a*) and at different times after removal of potassium from the bathing fluid (*b*—*f*). It can be seen that the critical strength of the stimulus required to evoke an impulse increased gradually with time. The decrease

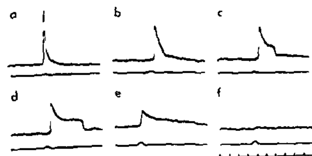


Fig. 3. Increase in threshold after removal of potassium. Spindle tested at different intervals with brief stretch (lower trace). Record *a* normal Ringer, *b* 27 min, *c* 30 min, *d* 36 min, *e* 40 min and *f* 95 min after removal of potassium. Time marks 5 ms.

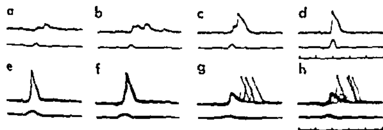


Fig 7 Dec... al phase of recovery in ... 00 min Records *a*—*d*, ... repetitive brief stretches ... —*h*, 9 min after return

Recovery

After return to normal Ringer the spontaneous activity usually reappeared within a few minutes The first spikes to appear had a prolonged plateau In the following minutes the plateau decreased rapidly in duration and disappeared The behaviour of the spindle in the early phase of recovery closely resembled that in the late phase of exposure to potassium free Ringer Hence the shape and size of the response to a brief stretch were greatly dependent on the strength of the stretch At low levels of stretch the response often consisted of two or three potential elevations as is illustrated by records *a*—*c* in Fig 7 With increasing strength of stretch the small potentials usually merged into a spike with a typical plateau (*d*) With further increase in amount of stretch this impulse remained unaltered The spike could be decomposited into smaller unitary responses by subjecting the spindle to brief repetitive stretches at 10—20/sec (records *e*—*h* in Fig 7)

The initial recovery of the impulse activity was generally very rapid and the plateau disappeared within 8—10 min after return to normal Ringer However the original amplitude and time course of the impulse was usually not restored until



Fig 8 Recovery of spindle after exposure to potassium free Ringer *a* 6 min *b* 16 min *c* 23 min after return to normal Ringer Time marks 50 ms

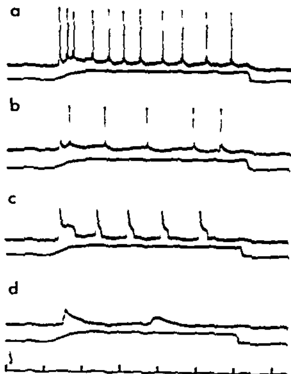


Fig 6 Effect of potassium removal on response of spindle to linearly rising stretch Record *a* normal Ringer *b* 10 min *c* 35 min *d* 50 min after removal of potassium Time marks 50 ms

breakdown of the impulse activity This effect appeared with prolonged soaking in potassium free solution and could be demonstrated by varying the strength of the stretch or by subjecting the spindle to repetitive stretching Similar effects were seen in the early stage of recovery following return to normal Ringer as will be described below

To test the effect on the dynamic and static response the spindle was subjected to linearly rising stretches of different velocities The records in Fig 6 illustrate the results from one of these experiments Record *a* shows the response to a relatively fast rising stretch when the spindle was kept in normal Ringer About 10 min after removal of potassium the same stretch gave a considerably weaker response At this stage the spikes were diminished in amplitude but there was yet no marked change in their configuration Later the plateau phase began to appear and the stretch now gave a response consisting of a series of heart like action potentials (*c* It may be noticed that the initial spike had a plateau of longer duration than the following static spikes This was a constant finding in all experiments (*cf* Fig 5) With still more prolonged soaking the static spikes changed into small wave like potentials and finally all signs of conducted activity disappeared At this stage a sustained potential could still be obtained as has been described earlier (Husmark and Ottoson 1971 *b*) This response represented the non conducted activity of the sensory endings It would thus appear that the effect of potassium removal on the axon develops more rapidly than the effect on the sensory endings enclosed by the capsule

haeuser (1962 *a b, c*) Later studies on giant axons perfused with solutions with little or no potassium have provided new and interesting aspects on the complex interaction between potassium and sodium (Narahashi 1963, Baker *et al* 1964) The action of potassium lack on the time course and shape of the impulse of the afferent fibre of the frog spindle is almost identical with that observed on the perfused giant axon (Baker *et al* 1964) It also closely conforms to the effect of scorpion venom (Adam *et al* 1966) and TEA (Schmidt and Stämpfli 1966) on myelinated fibres In all these cases the nerve impulse develops into a heart like action potential. There is strong evidence suggesting that this change is due to a delayed sodium inactivation It would appear plausible that an analogous mechanism is responsible for the production of the prolonged heart like action potential of the axon of the spindle

The decrease in responsiveness of the spindle to stretch after removal of potassium could be due either to an increased threshold of the nerve fibre or a reduction of the receptor potential produced by stretch The experimental results suggest that both these factors contribute An increased threshold of the fibre is suggested by the finding that the reduced responsiveness appeared rapidly after removal of potassium whereas the diminution of the receptor potential developed considerably more slowly (Husmark and Ottoson 1971 *b*) It would thus appear that the initial fall in sensitivity after potassium removal may be attributed to the effect on the afferent fibre while in the later stages both factors contribute There seems to be reason to believe that these effects are related to a reduction of the resting potential of the fibre and the endings This view is supported by the finding that the fall in responsiveness was associated with a progressive reduction in height and final disappearance of the impulses It also gains support from observations on the perfused giant axon showing that removal of potassium causes a reduction of the resting potential and affects the critical potential for activation of the axon (Baker *et al* 1964) The fact that there was a simultaneous increase in spontaneous activity and a decrease in responsiveness to stretch in the early stage of potassium lack suggests that other changes than those mentioned above are induced when potassium ions are removed The nature of these changes can not be resolved at the present time

In summary the results show that potassium ions are of critical importance for the transducer action and the impulse generation of the spindle The precise nature of the changes in ionic mechanisms induced by removal of potassium can not yet be determined It appears most likely however that the observed effects may be explained by a delayed sodium inactivation and a reduction of the resting potential of the afferent fibre and the sensory endings

The authors are greatly indebted to Prof B Frankenhaeuser and Dr G M Shepherd for valuable discussions during the preparation of this manuscript We also wish to thank Miss Olga Popoff for unfailing assistance

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after 30—40 min in normal Ringer. A typical example of the restoration of the response is shown in Fig. 8. In the early phase of recovery a steplike test stretch evoked only one initial prolonged impulse followed by a few wave like potentials during maintained stretch (*a*). About 10 min later a discharge of more normally looking spikes was obtained (*b*). Between these impulses there were small abortive spikes (*cf.* Katz 1950, Ottoson and Shepherd 1970) indicating an incomplete synchronization of the activity of the nerve branches within the spindle. These small abortive spikes usually disappeared after 20—30 min in Ringer. Although the spikes at this stage of recovery appeared almost normal the responsiveness of the spindle was not restored as shown by the low frequency of the static discharge. Two hours after return to Ringer recovery was still not complete. This indicates that prolonged exposure of the spindle to potassium lack produces irreversible alterations in the responsiveness of the spindle and in the impulse generating mechanisms.

Discussion

The present results show that the behaviour of the muscle spindle is markedly modified by removal of potassium from the external solution. The effect on the impulse activity of the spindle is characterized by an increased spontaneous activity, a prolongation and decrease of the action potential followed by a final blocking of the conducted activity. These changes appeared with striking regularity and with little time difference in different preparations. The finding that with prolonged exposure of the spindle to potassium free Ringer the receptor potential persisted after the conducted activity had disappeared suggests that the endings are less susceptible to lack of potassium than the stem fibre and its branches. This difference might be explained by the fact that the endings are protected by the outer and inner capsule of the spindle and therefore slowly affected by ionic changes in the bathing fluid. An interesting point is that the receptor potential obtained after disappearance of the conducted activity differs in shape from the normal receptor potential. This change has been described in a previous paper (Husmark and Ottoson 1971 *a*).

An increase in resting discharge of the spindle after removal of potassium was observed by Matthews in his study in 1931. He also noted that the discharge in potassium free Ringer was very regular. Similar effects have been reported from experiments on myelinated nerves treated with TEA (Burke, Katz and Machne 1953) and in studies on giant axons perfused with low potassium solutions (Narahashi 1963, Baker *et al.* 1964). Burke *et al.* (1953) suggested that the increased resting activity was due to the prolongation of the negative after potential. A similar explanation may also be valid for the effect on the spindle activity. In addition to the effect of the prolonged negative after potential a decrease of the resting potential (*cf.* Baker *et al.* 1964) might also have contributed to the increase in spontaneous activity.

The quantitative contribution of potassium to the production of the nerve impulse has been established by the work of Hodgkin and Huxley (1952) and Franken

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The Effect of a Noradrenaline Liberator (4, alpha-dimethyl-meta-tyramine) on Reflex Transmission in Spinal Cats

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Abstract

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... -methyl-meta-tyramine (H 77/77), ... reflex ... The ... FRA ... energic ... which ... tyrosine ... eration ... FRA ... on it is ... ones deprived of NA ... functions of the NA ... terminals

An i.v. injection of L-3,4-hydroxyphenylalanine (DOPA) gives a profound functional change in the spinal cord which includes a radically different pattern of reflex effects to α- and γ-motoneurones as well as to primary afferent terminals (Lundberg 1966, Grillner 1969). Andén *et al.* (1966 a) proposed that these effects were produced by transmitter liberation from noradrenergic terminals known to belong to reticulospinal neurones (Carlsson *et al.* 1964, Dahlström and Fuxe 1965).

There is already substantial support for this hypothesis. For example, the effect of DOPA shows a 10 fold potentiation after inhibition of monoamine oxidase (MAO), which inactivates noradrenaline (NA) and the effect by DOPA is completely antagonized by the adrenergic α-receptor blocker phenoxybenzamine (Andén *et al.* 1966 a). Furthermore, DOPA has no effect after inhibition of DOPA decarbo

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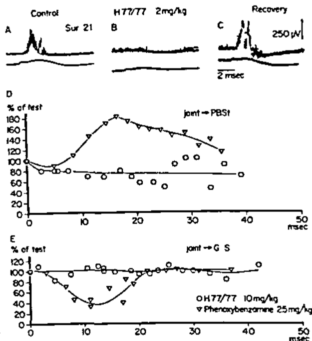
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Fig 1 The effect of H 77/77 on ventral root discharges evoked by stimulation of the sural nerve Upper traces are from the ventral root S1 and lower traces from the L7 dorsal root entry zone A was taken immediately before and B 14 min after an i v injection of 2 mg/kg H 77/77 C shows the recovery 3 5 h after the injection The graphs in D and E illustrate the effect of a conditioning volley in the posterior knee joint nerve on monosynaptic test reflexes of flexors (PBSt) and extensors (G-S) Originally there was flexor facilitation and extensor inhibition (not illustrated) but these effects were virtually abolished by 10 mg/kg H 77/77 (open circles) The facilitatory and inhibitory effects reappeared a few minutes after injection of 25 mg/kg phenoxybenzamine (triangles) The effects of the conditioning volley are expressed as percentages of the unconditioned test (ordinate) against the time interval between the conditioning and test stimulus (abscissa) The strength of the stimuli expressed in multiples of threshold for the nerves is indicated in each record



Results

...*otoneurones* In acute spinal cats volleys in cutaneous and high ...fferents evoke excitation in flexor and inhibition in extensor ...effects are mediated by the short latency reflex pathways from (FRA) The effect of a drug on the efficacy of transmission ...ventral root discharges evoked from the FRA and ... monosynaptic test reflexes to flexors and ex

...r H 77/77 depresses transmission in these ...A However, an intravenous injection of ... an increased motoneuronal excitability ... the ventral roots and considerably in ... This effect had a very rapid ... after the injection It is therefore ...und and not related to its more ... from the FRA Evidence for the ... that the ventral root discharge ... after 2 mg/kg H 77/77 ... this reflex discharge it is

(the enzyme responsible for the synthesis of dopamine (DA) from DOPA) which shows that DOPA does not act directly but through catecholamine formation (Andén *et al.* 1966 b). The effect of DOPA is also changed after inhibition of dopamine β-hydroxylase (which converts DA to NA) (Jurna and Lundberg 1968). Inhibition of this enzyme does not influence the effect of a single injection of DOPA but a second injection of DOPA was completely ineffective. The explanation proposed by Jurna and Lundberg (1968) is that DA formed by the first injection of DOPA displaces NA from its stores and that as a consequence NA is liberated from the terminals. However, since the stores of NA in the terminals are depleted and no new NA can be formed from DA, the second injection of DOPA is ineffective.

Despite all these findings it is nevertheless somewhat unsatisfactory to base conclusions regarding the function of the descending NA pathways exclusively on the analysis of the effect of DOPA. It is difficult to exclude entirely that DOPA may be acted upon by enzymes located outside NA nerve terminals. Accordingly, it was desirable to investigate the effect of NA liberation by some other process not involving administration of a precursor. We have abstained from the most direct experimental approach of electrical stimulation of the descending NA neurones because it is difficult to avoid coactivation of other descending pathways with similar effects (*cf.* Lundberg 1966). Rather we attempted to elucidate the problem by investigating the effect of drugs which liberate NA without being precursors. The most well known of these drugs is reserpine, which liberates both NA and 5-HT. Reserpine does in fact (after MAO inhibition) give similar effects as DOPA (Andén *et al.* 1964, Engberg *et al.* 1968), but this effect seemed to be caused entirely by liberation of 5-HT since it is reversed by 5-HT receptor blockers but not by phenoxybenzamine (Engberg *et al.* 1968).

Recently Carlsson *et al.* (1969, 1970) have reported that 4, α-dimetyl-meta-tyramine (H 77/77) releases NA but not 5-HT. It will be shown that this substance inhibits reflex transmission in the spinal cord in the same way as DOPA and that the effect is completely reversed by blockers of adrenergic receptors.

Methods

All experiments were carried out on unanesthetized anemically decorticated cats (*cf.* Andén *et al.* 1966 a). The spinal cord was sectioned at the lower thoracic level; in chronic spinal cats this transection was made 2–4 weeks before the acute experiment. The animals were immobilized with gallamine triethiodide (Flaxedil, M & B) and artificially ventilated. The blood pressure was measured continuously in all the experiments. The technique of conditioning [illegible] reflexes [illegible] potentials and the discharge in ascending path[illegible] [illegible] 1959, Carpenter *et al.* 1963, Laporte, Lundberg [illegible]

The following drugs were used: H 77/77 (4, α-dimethyl-meta-tyramine), Hassle Ltd, Göteborg, H 44/68 (inhibitor of tyrosine hydroxylase), Hassle Ltd, Göteborg; phenoxybenzamine (dibenzyline), Smith, Kline and French Labs, Philadelphia; reserpine (Serpasil), Swedish CIBA Ltd., chlorpromazine (Hibernal), Leo Ltd, Helsingborg.

Abbreviations: G-S, gastrocnemius-soleus nerve; joint, posterior knee joint nerve; Sur, sural [illegible]

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VSCT, ventral spinocerebellar tract; SCT, spinocervical tract.

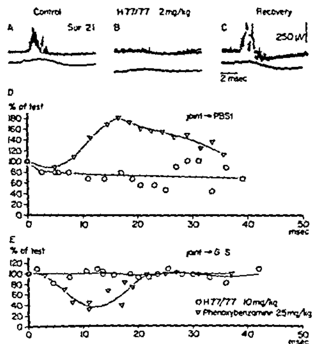


Fig 1 The effect of H 77/77 on ventral root discharges evoked by stimulation of the sural nerve. Upper traces are from the ventral root S1 and lower traces from the L7 dorsal root entry zone. A was taken immediately before and B 14 min after an i.v. injection of 2 mg/kg H 77/77. C shows the recovery 3.5 h after the injection. The graphs in D and E illustrate the effect of a conditioning volley in the posterior knee joint nerve on monosynaptic test reflexes of flexors (PBSt) and extensors (G-S). Originally there was flexor facilitation and extensor inhibition (not illustrated) but these effects were virtually abolished by 10 mg/kg H 77/77 (open circles). The facilitatory and inhibitory effects reappeared a few minutes after injection of 25 mg/kg phenoxybenzamine (triangles). The effects of the conditioning volley are expressed as percentages of the unconditioned test (ordinate) against the time interval between the conditioning and test stimulus (abscissa). The strength of the stimuli expressed in multiples of threshold for the nerves is indicated in each record.

Results

Transmission to motoneurones. In acute spinal cats volleys in cutaneous and high threshold muscle afferents evoke excitation in flexor and inhibition in extensor motoneurones. These effects are mediated by the short latency reflex pathways from the flexor reflex afferents (FRA). The effect of a drug on the efficacy of transmission in these pathways can be tested on ventral root discharges evoked from the FRA and on the facilitation and inhibition of monosynaptic test reflexes to flexors and extensors by FRA volleys.

It will be shown that the NA liberator H 77/77 depresses transmission in these short latency reflex pathways from the FRA. However an intravenous injection of H 77/77 in doses 1—5 mg/kg also produced an increased motoneuronal excitability as evidenced by both a resting discharge in the ventral roots and considerably increased monosynaptic reflexes to flexors and extensors. This effect had a very rapid onset and reached a maximum within one minute after the injection. It is therefore thought to be caused by a direct action of the compound and not related to its more slowly developing inhibitory effect on transmission from the FRA. Evidence for the latter effect is given in Fig 1. Records A and B show that the ventral root discharge evoked by a volley in the sural nerve is completely depressed after 2 mg/kg H 77/7. Since the direct effect of the drug would tend to increase this reflex disch

clear that H 77/77 depresses transmission in the excitatory pathway from the FRA to motoneurones Correspondingly there was a decrease of the facilitatory and inhibitory effects from the FRA on the monosynaptic test reflexes sometimes to the extent that they were abolished (Fig 1, D F) The onset of the action of H 77/77 was slow the depression increasing gradually to reach a maximum about 10 minutes after the injection Full recovery occurred after 2—4 h (record C)

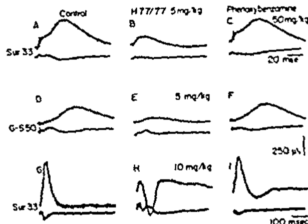
At any state the inhibitory effect on transmission from the FRA could be completely reversed by an intravenous injection of the adrenergic receptor blocker phenoxybenzamine (25 mg/kg) This is illustrated in Fig 1 by the reappearance of the facilitatory (D) and inhibitory (E) actions from high threshold joint afferents on the monosynaptic test reflexes from a flexor and an extensor muscle Chlorpromazine 3 mg/kg was also effective in removing the effect of H 77/77

The dose of H 77/77 required to produce these effects was smaller than that giving NA depletion in rats (Carlsson *et al* 1968 1969) However, in cats 5 mg/kg H 77/77 reduced the NA concentration of the spinal cord to about half the normal value without any change in the 5 HT concentration (Corrodi personal communication) Accordingly the effects by H 77/77 may be due to liberation of NA

The depression of short latency transmission from the FRA closely resembled that found after an injection of DOPA but in another respect the action of these drugs differed The late longlasting flexor reflex which is such a characteristic finding after an intravenous injection of DOPA (Anden *et al* 1966 a Jankowska *et al* 1967 was never observed after an injection of H 77/77 not even in those experiments when the early ventral root discharge from the FRA was completely depressed This difference will be considered in the discussion

Transmission to ascending pathways The effect of H 77/77 on the transmission to ascending pathways was similar to that after administration of DOPA A dose of 5 mg/kg H 77/77 produced a profound depression of the late mass discharge from high threshold muscle afferents and cutaneous afferents in both the ipsilateral and the contralateral spinal half However similarly to DOPA H 77/77 had neither any effect on the monosynaptic transmission from group I muscle afferents to the DSCT and the VSCT nor on monosynaptic transmission from the cutaneous afferents to the SCT

Transmission to primary afferent terminals In important respects the effects of H 77/77 on transmission to primary afferent terminals also resemble those of DOPA Fig 2 shows DRPs evoked by volleys in the FRA before and after 5 0 mg H 77/77 There was an effective depression of the DRPs from cutaneous (A and D) and high threshold muscle (B E afferents Note that in record B the second component of the DRP evoked from cutaneous afferents is almost abolished after H 77/77 whereas the first component is much less changed (*cf* Anden *et al* 1966 a) It has been shown that only the second component is transmitted by the pathways from the FRA whereas the first one is mediated by a special reflex pathway from cutaneous afferents (Carpenter *et al* 1963) The time course of the effect on transmission to primary afferent terminals resembled that on transmission to motoneurones (graph in Fig 2)



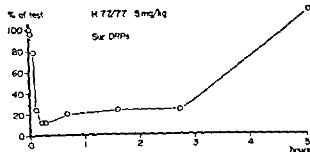


Fig 2 The effect of H 77/77 on DRPs recorded from the most caudal filament of dorsal root L6 (upper traces), incoming volleys from the L7 dorsal root entry zone (lower traces) A D G were taken before application of H 77/77 B and E after 5 mg/kg and H after 10 mg/kg H 77/77 C, F and I were taken a few min after 50 mg/kg phenoxybenzamine High threshold afferent fibres were stimulated in the sural nerve (Sur) and gastrocnemius soleus nerve (G-S) Single volleys were used in A F, a short train of volleys in G-I In the diagram percentages of the test amplitude of the DRPs evoked by stimulation of the sural nerve were plotted against time interval after injection of 5 mg/kg H 77/77

Record C and F show the almost complete recovery of the DRPs after 50 mg/kg phenoxybenzamine

The late longlasting DRPs usually evoked from the FRA after DOPA were only very rarely found after H 77/77 Nevertheless when they were evoked, the time course was similar to that found after DOPA Anden *et al* (1966 c) have shown that after an injection of DOPA volleys in the FRA sometimes produced a positive DRP In this respect H 77/77 was even more effective than DOPA When higher doses (10 mg/kg) were given a train of volleys in the FRA produced positive DRPs of large amplitude (Fig 2 H) Also this effect was fully reversed by phenoxybenzamine (I)

Anden *et al* (1966 c) have shown that the late positive DRP sometimes evoked from the FRA after DOPA is caused by a selective hyperpolarization in Ia afferent terminals They propo
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In experiments in which positive DRPs were evoked by volleys in the FRA after injection of H 77/77 it was observed that the DRPs elicited by a short train of impulses in Ia afferents were depressed by the injection A corresponding

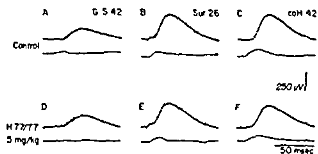


Fig 3 Lack of effect by H 77/77 on DRPs after pretreatment with reserpine and H 44/68 injected 48 h (100 mg/kg and 0 5 mg/kg) and 24 h (50 mg/kg and 0 25 mg/kg) before the acute experiment Upper traces DRPs recorded from the most caudal dorsal rootlet in L6 Lower traces, incoming volleys recorded from the L7 dorsal root entry zone A B and C were taken immediately before and D, E and F 20 min after 5 mg/kg H 77/77

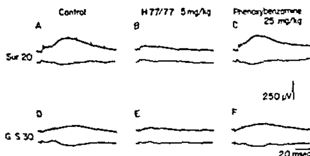
was also seen after DOPA, particularly in cats pretreated with the MAO inhibitor Nialamid (unpublished observations) Such a depression of the Ia DRP would rather be expected if there is a spontaneous activity in the neuronal pathway mediating depolarization between Ia afferents

After NA depletion Carlsson *et al* (1969) and Carlsson *et al* (1970) have shown that H 77/77 depletes the central stores of NA but not of 5 HT If the effects produced by H 77/77 are due to increased liberation of NA from the terminals of monoaminergic descending pathways, then they should not appear after pretreatment with drugs depleting the stores of NA To test this possibility the cats were pretreated with reserpine and the tyrosine-hydroxylase inhibitor H 44/68 (Corrodi and Hanson 1966) in order to empty the NA stores and block the synthesis of NA 100 mg/kg H 44/68 and 0 5 mg/kg reserpine was injected intraperitoneally 48 h, and half of this dose 24 h before the acute experiments The records in Fig 3 illustrate that after such combined pretreatment, 5 mg/kg H 77/77 had very little, if any, effect on the DRPs evoked from the high threshold muscle afferents and the cutaneous afferents

Chronic spinal cats All noradrenergic terminals in the spinal cord seem to belong to descending fibres originating in the brain stem (Carlsson *et al* 1964) Accordingly it might be expected that a drug acting by liberation of NA should be without effect after degeneration of the descending NA fibres However, it is recalled that DOPA is effective also in chronic spinal cats We have investigated also the effects of H 77/77 in four chronic spinal cats in which the spinal cord was transected at the lower thoracic level (Th 11—12) The effects on transmission from the FRA to motoneurones and to the primary afferent terminals was tested 2—4 weeks after the transection

Fig 4 shows that 5 mg/kg H 77/77 effectively depressed the DRPs both from cutaneous (A, B) and from high threshold muscle afferents (D, E) As in the acute spinal cats this effect could be reversed by phenoxybenzamine (C—F) Chlorpromazine was also found to be effective in antagonizing the action of H 77/77, but after a dose of 3 mg/kg used in our experiments, the recovery of the DRPs was only partial

Fig 4 The effect of H 77/77 on DRPs evoked from the cutaneous and high threshold muscle afferents in a chronic spinal cat The transection at Th 11 was made 2 weeks before the acute experiment Upper traces DRPs recorded from the most caudal dorsal rootlet L6 Lower traces incoming volleys recorded from the L7 dorsal rot entry zone A and D were taken immediately before and B and E 5 min after 5 mg/kg H 77/77 Recordings C and F were taken a few minutes after 25 mg/kg phenoxybenzamine



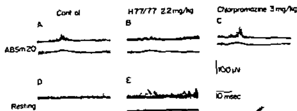
The effect of H 77/77 on the transmission from FRA to motoneurones in chronic spinal cats also resembled that seen in the acute spinal animals As demonstrated in Fig 5 the ventral root discharge in high threshold muscle afferents nearly disappeared when 2 2 mg/kg H 77/77 was applied (B), while the resting activity in the ventral root was markedly increased (E) Chlorpromazine, 3 mg/kg was effective in removing the depressant action on ventral root discharge (C)

Hence H 77/77 produces changes in the transmission of the short latency reflex pathway from the FRA when descending pathways are degenerated Therefore another mechanism than transmitter release from NA terminals has to be considered to account for the action of H 77/77 in chronic spinal cats

Discussion

Compound H 77/77 has the same depressing effect as DOPA on transmission in the short latency pathway from the FRA to primary afferent terminals motoneurones and ascending pathways Since all the effects like those of DOPA can be completely reversed by the noradrenergic α blocker phenoxybenzamine and chlorpromazine

Fig 5 The effect of H 77/77 on ventral root discharges evoked from high threshold muscle afferents in chronic spinal cat Transection at Th 11 was made 2 weeks before the acute experiment The upper traces are from the S1 ventral root and the lower from the L7 dorsal rot entry zone A was recorded immediately before B 8 min after 2 2 mg/kg H 77/77, and C 18 min after 3 mg/kg chlorpromazine D represents resting ventral root discharges before and E 4 min after 2 2 mg/kg H 77/77 All records consist of 3—4 superimposed traces



(Anden *et al* 1966 a, b) it can be postulated that they are exerted via NA receptors
On the other hand the long latency flexor reflex which is such a characteristic feature after DOPA was never observed after H 77/77 and also the late DRPs were usually missing This finding however does not contradict the postulate that administration of H 77/77 produces activation of the NA receptors The late reflex after DOPA occurs as a release phenomenon secondary to inhibition of the short latency FRA pathways and can be completely blocked by a very small dose of anesthesia which does not influence the effect of DOPA transmission in the short latency pathways (Anden *et al* 1966c Jankowska *et al* 1966) Accordingly, the absence of the late reflex effects after H 77/77 may be due to a direct depressing action of the drug on the multineuronal pathways mediating the late reflexes It is also assumed that the resting discharges in the ventral roots which appeared very quickly after injection of H 77/77 are caused by a direct action of the drug not related to stimulation of NA receptors

Carlsson *et al* (1968 1970) have shown that H 77/77 gives a selective depletion of the NA stores in the brain by displacement of the endogenous NA It would therefore be expected that the compound is taken up in the terminals of the descending NA fibres in the spinal cord and liberates NA from them If this is the mechanism by which it depresses reflex transmission then the effect of H 77/77 would depend on pre existing stores of NA in the terminals Thus our finding that H 77/77 has no effect after NA depletion following treatment with H 44/68 and reserpine is most easily explained by the depletion of NA produced by these drugs The alternative possibility that H 77/77 acts by direct stimulation of NA receptors seems to be excluded by the very finding that it does not act after H 44/68 and reserpine as it has been shown that these drugs do not block central NA receptors (Anden *et al* 1970) It may still be argued that H 77/77 acts in the acute spinal cat through a metabolite formed by the action of DA β hydroxylase This possibility cannot be excluded by the absence of effect in cats pretreated with H 44/68 and reserpine because DA β hydroxylase may be located inside the storage granulae and be less accessible to the compound after reserpine which is supposed to block the amine uptake by the storage granulae However an action through a hydroxylated metabolite is unlikely since it has recently been shown (in rats) that H 77/77 is not metabolized by DA β hydroxylase (Meisch and Waldeck personal communication)

Since all monoaminergic terminals belong to descending pathways (Carlsson *et al* 1964, Dahlstrom and Fuxe 1965) it is possible to perform control experiments regarding the effect of a transmitter liberator on chronic spinal cats Such experiments proved their value in the analysis of the effects produced by the monoamine liberator reserpine When MAO was inhibited reserpine depressed reflex transmission in acute spinal cats while being completely ineffective in chronic spinal cats (Engberg *et al* 1968) Accordingly it was postulated that the reserpine induced effect is caused by transmitter liberation from monoaminergic nerve terminals but as mentioned in the introduction they seem to belong to 5 HT pathways

The NA liberator H 77/77 on the other hand acts also in chronic spinal cats

Seemingly this finding could be taken as evidence against the view that it does act in the acute spinal cats by liberating transmitter from noradrenergic terminals However it should be realized that spinal segments below the section may undergo profound changes after degeneration of the descending pathways Thus it cannot be excluded that the neurones deprived of their noradrenergic innervation have developed a sensitivity to compound H 77/77 A somewhat analogous mechanism was considered by Anden *et al* (1966 b) in an attempt to explain the effect of DOPA in chronic spinal cats

Alternatively DOPA may act through the formation of NA also in the chronic spinal cats provided the enzymes required for its formation decarboxylase and DA β-hydroxylase, have remained in sufficient amounts in degenerating terminals or have been taken up by neighbouring glia cells However, the presence of these enzymes in neighbouring cellular elements could explain the effect of H 77/77 only if it is assumed that they occur together with storage granulae containing NA, which can be released by the compound It has been shown that such a mechanism does exist at the neuromuscular junction In the denervated muscle the terminal Schwann cells absorb the remnants of the motor nerve terminals and also take over some of its function including the ability to synthesize and release acetylcholine (Birks Katz and Miledi 1960, Miledi and Slater 1968)

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Interaction between the Fastigial Pressor Response and the Baroreceptor Reflex

By

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Abstract

LISANDER, B and J MARTNER *Interaction between the fastigial pressor response and the baroreceptor reflex* Acta physiol scand 1971 *83* 505—514

Experiments were performed on chloralosed cats with recording of blood pressure heart rate and [illegible] blood baroreceptor unloading or to somatic pressor afferent stimulation indicating that this area did not exert any tonic influence on the centres conveying such reflexes Evidence is presented for a differentiated interaction between the baroreceptor inhibitory reflex and the fastigial excitatory response with a relatively stronger fastigial suppression of the reflex action on the heart than on the vascular bed This differentiation involves an element of central suppression of the reflex cardiac control though it is partly a consequence of the different neuroeffector characteristics of heart and vessels

Stimulation of parts of the feline fastigial nucleus sometimes elicits very marked blood pressure rises (Miura and Reis 1969 Achari and Downman 1970) The efferent peripheral pathways appear to be primarily the sympathetic vasoconstrictor nerves, the contribution from the adrenals is negligible and cardioacceleration does not always occur but if so it is claimed to be solely adrenergically mediated (Achari and Downman 1970)

Such a pressor response must inevitably cause a baroreceptor activation and to judge from the literature opinions concerning the complex interaction between the fastigial excitatory influence and the baroreceptor reflexes diverge According to Miura and Reis (1969) the fastigial pressor response is abolished by destruction of the paramedian reticular nucleus which seems to receive heavy projections from both the carotid and the aortic baroreceptor pathways (Miura and Reis 1968 Crill and Reis 1968) This may suggest that the fastigial pressor response is brought a by 'switching off the baroreceptor reflexes On the other hand Achari and

man (1970) could still elicit fastigial pressor responses after denervation of all peripheral baroreceptor areas It was, however, clear from their experiments that fastigial stimulation could almost completely annul reflex bradycardia due to carotid sinus distension, vagal afferent stimulation or phenyl diguanide administration. These observations suggest an abrogation of the mentioned reflex influences during fastigial stimulation but it is surprising that no inhibition of resting vagal tone could be observed

In the course of recent studies, dealing with the cerebellar interference with the cardiovascular defence reaction (Lisander and Martner 1971), some observations were made that directed our interest also to the interaction between the cerebellar responses and the baroreceptor reflexes Below evidence will be presented for a differentiated interaction between the fastigial pressor response and the baroreceptor reflexes

Methods

Experiments were performed on 30 cats of both sexes After induction with ether, lght anesthesia was maintained by i.v. administration of chloralose 30—50 mg/kg b.wt

For fastigial stimulation the animal's head was fixed in a Horsley Clarke stereotaxic apparatus and the occipital bone was freed in the midline Following trephination and gentle removal parts of the tentorium cerebelli sharp stainless steel monopolar electrodes were inserted into fastigial nuclei using Horsley Clarke coordinates Square wave pulses were delivered by a ...ulator allowing for constant current stimulation at intensities of 0.05—0.4 mA and ...rations of usually 1 ms the frequency being varied within wide limits Cerebellar lesions were ...ade by 1 2 mA anodal current applied for 30 s (The stimulator was constructed by civ.ing L Stage To check the completeness of cerebellar lesions stimulating pulses of up to 1 mA were used Hypothalamic defence area stimulations with a Grass S5 stimulator were often performed as well for comparison and to check the patency of the central nervous control of vascular beds under study After each experiment the head of the animal was perfused with saline followed by formaldehyde and relevant parts of the central nervous system were paraffin embedded sectioned and stained by the Nissl technique For interruption of spinal pathways the spinal cord was exposed and transected at Th1

Heparin was given as an anticoagulant[1] Blood pressure was measured through a catheter in the femoral artery connected to a Statham P23 AC transducer writing on a Grass polygraph. Heart rate was recorded by connecting the pressure recording amplifier to a Grass Tachograph unit Muscle blood flow was measured as the outflow from the deep femoral vein the paw circulation being excluded by a tight ligature at the ankle After passing a closed optical drop recorder device operating an ordinate writer the blood was returned to the animal *via* one of the femoral veins In a few experiments the leg was skinned and the calf muscles completely isolated a hole being drilled in the femur and the bone marrow plugged Concomitantly with the muscle blood flow either intestinal renal or cutaneous blood flow were recorded as well with the same technique When intestinal blood flow was to be measured the intestines with exception for a 20—30 g jejunal segment were extirpated after which the venous outflow from the remaining segment passed another drop recorder returning to the animal *via* the portal vein Skin blood flow in the hindpaw was similarly recorded from a cannula in the great saphenous vein other major veins being ligated and renal blood flow from the renal vein care being taken not to damage the regional periarterial nerve supply during the cannulations In a few experiments the adrenals were exposed transabdominally and ligated in the majority of the cases however the time course of the responses was used as the criterion that they were neurogenic

The vagus nerves were dissected free in the neck and placed on ligatures so that they could be cut in the course of the experiment Vagal afferent stimulation was performed with an ...nular silver electrodes and a Grass S5 stimulator For baroreceptor stimulations the carotid sinus region was gently dissected free and partly isolated and connected to one of the femoral arteries by a tube This tube could by means of a sigma motor pump deliver various levels of

[1] We are indebted to Vitrum Stockholm for generous supply of heparin

pulsating pressure to the carotid sinus or it could transmit the ambient blood pressure of the animal This sinus pressure was measured [illegible]
AC transducer In some experiments th[illegible]
was connected to the external jugular [illegible]
that the mean sinus pressure could be [illegible]
nerve was freed and sectioned Barorec[illegible]
the common carotid arteries or of the perfusion tube

Some of the animals received gallaminetriethiodide (Flaxedil®, May and Baker, 2—4 mg/kg) to eliminate cardiovascular effects that could be induced secondary to somatomotor changes Constant artificial respiration was then maintained by means of a respiration pump Atropine (0.5—1 mg/kg was given i v to block vagal effects on the heart For β adrenergic blockade alprenolol[2] (0 5 mg/kg b w i v) was given

Results

In order to assess whether connections via the fastigial nuclei are of importance for the reflex cardiovascular responses induced by carotid occlusion or by stimulation of somatic "pressor" afferents (see Johansson 1962), these responses were compared before and after electrolytic lesions of the fastigial pressor area To avoid time-dependent changes, the operative preparation was kept at a minimum and only blood pressure and heart rate were therefore recorded The fastigial pressor area was localized bilaterally by topical stimulations, the electrodes being subsequently used for producing lesions The completeness of the lesions was checked by repeated stimulations at high strengths It was found that destruction of the fastigial pressor areas affected neither the above mentioned reflex responses nor the 'resting' levels of blood pressure and heart rate

Stimulation within the rostral pole of the fastigial nucleus regularly evoked pressor responses that sometimes were as marked as 130 mm Hg As shown also by Achari and Downman (1970), there was a simultaneous increase in vascular resistance in skeletal muscle, skin intestine and kidney a rise in heart rate was usually also observed, though occasionally it was reduced instead

An attempt was made to obtain quantitative information about the degree of constriction in the various vascular beds For this purpose the regional resistance increases caused by fastigial stimulation were compared to those induced reflexly by complete or partial occlusion of the common carotids in vagotomized cats The blood pressure responses usually attained their maxima at lower frequencies of fastigial stimulation than did the regional resistance responses (see below Fig 3) In most experiments it was possible to adjust the stimulation frequency so that the fastigial blood pressure responses closely mimicked the reflex blood pressure responses to baroreceptor unloading thus enabling a direct comparison of the regional increases of flow resistance Although the magnitude of the circulatory response quite naturally varied between experiments the pattern of engagement of the different parallel coupled circuits was essentially the same whether the baroreceptor restraint was reduced or a fastigial stimulation was performed In both cases the vasoconstrictions in the skeletal muscles and the intestines usually exceeded those in the renal [illegible] cutaneous vascular beds

[2] We are indebted to AB Hassle Goteborg for generous supply of Aptin®

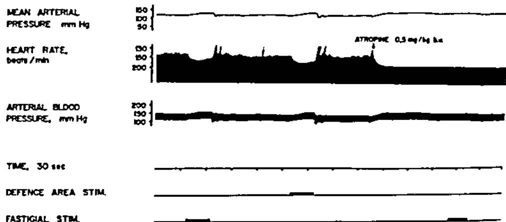


Fig 1 Cat 3 2 kg spinalized at Th 1 Defence area (80 imp/s 1 msec 4 V) and fastigial stimulations (30 imp/s 1 ms 0 3 mA) Note complete absence of circulatory changes upon stimulation after atropine administration

As mentioned the fastigial pressor responses usually involved a rise in heart rate, though only exceptionally reaching 250 beats/min while this level could easily be attained by hypothalamic defence area stimulation The cardioaccelerations remained after vagotomy or following atropine administration indicating that they were at least in part sympathetically mediated — To test whether any inhibition of vagal tone also contributed to the increase of the heart rate, experiments were performed in cats which were spinalized at Th 1, but transfused with dextran to keep the blood pressure at an almost normal level Fig 1 illustrates such an experiment Fastigial stimulation repeatedly induced cardioacceleration provided resting heart rate was below 195 beats/min These responses were abolished after atropine In two additional experiments the adrenergic supply to the heart was blocked by the β adrenergic blocking drug alprenolol (0 5 mg/kg b wt i v) Also here fastigial stimulation

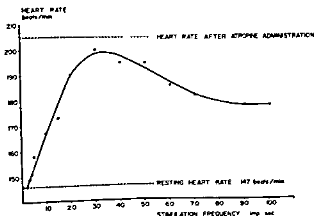


Fig 2 Same spinal cat as in Fig 1 Frequency response curve compiled from 15 fastigial stimulations (1 ms 0 3 mA) For further explanations see text

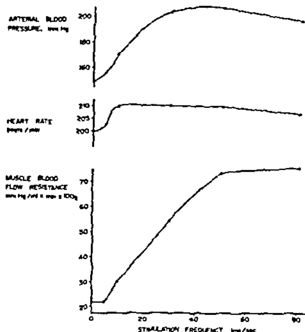


Fig 3 Cat 2 5 kg vagotomized Fastigial stimulations (1 ms, 0 3 mA) at varying impulse rates Stippled area indicates range of resting values For further explanations see text

produced cardioacceleration, which was abolished by atropine The frequency-response curve for this vagally mediated cardioacceleration, induced by fastigial stimulation, appears to be steep, reaching its maximum at fairly low rates of fastigial stimulation (Fig 2)

Fig 3 shows representative cardiovascular responses to fastigial stimulations at various frequencies in a vagotomized cat, where the cardioaccelerations must be induced *via* the sympathetic supply to the heart Here maximal heart rate responses were attained even at frequencies as low as 10 imp/s while maximal blood pressure responses and muscle resistance increases were reached at about 30—40 and 50—80 imp/s, respectively

If fastigial stimulations at 50 imp/s were carried out during standardized baroreceptor activations, these stimulations moderately interfered with the reflex reductions in blood pressure and regional resistance, whereas the reflex inhibition of heart rate was almost completely abolished by the concomitant fastigial stimulation Fig 4 shows effects of a standardized increase of baroreceptor activity carried out alone (at zero fastigial stimulation) or superimposed on fastigial stimulations at increasing frequencies The reflex reduction of heart rate caused by carotid sinus distension in this vagotomized cat amounts to about 20 beats/min The reflex bradycardia is progressively reduced and ultimately abolished by fastigial stimulations at rising frequencies However, the same fastigial stimulations influence only to a fairly small extent the concomitant reflex reductions in blood pressure and in muscle flow resistance at the frequencies shown here On the other hand, when clearly

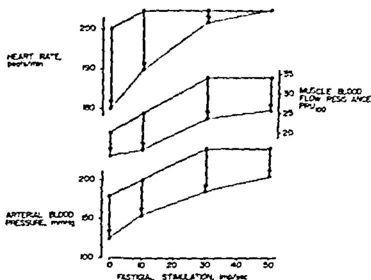
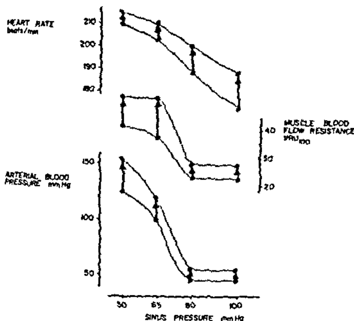


Fig 4 Cat 3 2 [illegible]
for inducing a [illegible]
0 1 mA were [illegible]
heart rate mus [illegible]
The heavy dots at the top of each area comprise the frequency response curve for fastigial [illegible]mulation when the carotid sinus is exposed to the pulsating systemic arterial pressure the [illegible]g lower dots illustrate the situation when the carotid sinus pressure is raised to a [illegible] extent simultaneously with the fastigial stimulation Note that the baroreceptor [illegible]ion on the heart rate causes a fall of about 20 beats/min when no fastigial stimulation is [illegible]rformed However when fastigial stimulations are added the reflex fall in heart rate becomes diminished and is abolished at 50 imp/s whereas the reflex fall in blood pressure and muscle flow resistance are only moderately affected

maximal frequencies of fastigial stimulation *e g* 80—100 imp/s (*cf* Fig 2—3), were used the reflex reductions in blood pressure and muscle flow resistance could be almost completely inhibited as well. A similar response pattern was found with intact vagus nerves other vascular beds behaved similarly to that of skeletal muscle

Fig 5 shows an experiment where the fastigial stimulation (50 imp/s is kept constant and where the extent of baroreceptor activation is instead varied As expected an increased carotid sinus distension causes a proportional reflex reduction in heart rate, but fastigial stimulation produces a rise that is more marked the more this rate is reflexly reduced to start with On the other hand, the augmentations in pressure and muscle flow resistance. caused by the standardized fastigial stimulation become more reduced the more intense the carotid baroreceptor activation is Thus, this figure illustrates in another way that the interaction between the baroreceptor inhibitory reflexes and the excitatory influence of fastigial stimulation differs quantitatively as to the nervous control of the heart and that of the vascular bed Essentially similar results were obtained when the vascular beds of intestine, skin or kidney were studied



5 Cat 2 2 kg intact vagi Right carotid sinus denervated perfusion of left sinus mean ...asinusoidal pressure varied From above downwards Heart rate muscle flow resistance ...od pressure Lower line in each shaded area shows the respective levels when graded activa...s of the carotid baroreceptors are induced alone top line the levels reached when a ...dardized fastigial stimulation (50 imp/s 1 ms 0 2 mA) is superimposed upon the same ...oreceptor activations

Discussion

It was considered of interest first to determine whether the fastigial nuclei are of importance for the reflex cardiovascular responses induced by carotid occlusion or by stimulation of somatic afferents (see Johansson 1962) No difference in these reflex responses was found after bilateral lesions of the fastigial nuclei suggesting that the fastigial neurons are not tonically involved in the modulation of these bulbar reflex mechanisms at least under the present experimental circumstances This by no means denies that fastigial neurons when activated may markedly modify the reflex mechanisms mentioned as will be further discussed below

In confirmation of the findings by Achari and Downman (1970) the present experiments show that topical stimulation of the fastigial pressor area produces a widespread systemic vasoconstriction It was however also attempted to determine whether this fastigial response also causes a differentiation in the vasoconstrictor discharge pattern to the various vascular beds as compared to that seen when the bulbar centres are relieved of the baroreceptor inhibitory influence For this purpose, the regional resistance increases caused by fastigial stimulation were compared with those caused by carotid baroreceptor unloading In cats during chloralose anesth... the latter procedure usually leads to a neurogenic vasoconstriction that is

marked in skeletal muscle less so in the intestine and negligible in the skin or the kidneys (Löfving 1961), at least as long as no other major reflex or central influence impinges upon the bulbar centres A certain chemoreceptor activation is usually also involved during these procedures for baroreceptor unloading but this added ex citatory drive on the bulbar centres appears to merely intensify the response pattern caused by a selective baroreceptor elimination *per se* (Löfving 1961) When the two procedures fastigial stimulation and baroreceptor unloading were made to produce comparable blood pressure rises the respective patterns of regional neurogenic con strictions were essentially similar in nature In other words fastigial stimulation produced a neurogenic vasoconstrictor pattern simulating that seen when the baro-receptor inhibitory influence was excluded or when this response was further potentiated by a chemoreceptor drive

The cardiac responses to fastigial stimulation were subject to further analysis. Usually but not always a cardioacceleration occurred, but it was then generally far less pronounced than that induced by hypothalamic defence area stimulation, even with intense fastigial stimulation The cardioacceleration was due to both an in creased sympathetic discharge and to a withdrawal of vagal tonic inhibition The latter mechanism was obvious from experiments on cats spinalized at Th 1, where fastigial stimulation still caused a rise in heart rate provided that the resting level below some 195 beats/min Also when a β adrenergic blocker with some inherent properties alprenolol (Åblad Brogård and Ek 1967) had been given cats with intact spinal cords fastigial stimulation increased the heart rate an fect that was blocked by atropine The absence of tachycardia upon stimulation after propranolol in the experiments by Achari and Downman (1970 might have been due to a reflex suppression of resting tonic activity in the vagal heart fibres in which case further inhibition is of course impossible.

If baroreceptor activation was induced during fastigial stimulation at 50 imp/s, the baroreceptor reflex influence still reduced blood pressure and regional flow re sistances markedly whereas the reflex inhibitory effect on heart rate was almost totally suppressed Such a differentiated interaction between the fastigial response pattern and the baroreceptor reflex influence is compatible with the finding by Achari and Downman (1970 that fastigial stimulation can completely extinguish baroreceptor reflex influences on the heart. However it also clearly indicates that *there is no generalized abrogation of the baroreceptor reflexes during fastigial stimu lation* as the reflex adjustment of the vascular bed appears to be far less affected. Moreover this differentiated interference is not an all or none phenomenon lower rates of fastigial stimulation only partly suppress the reflex cardiac inhibition, and likewise, "supramaximal rates of fastigial stimulation tend to suppress to some ex tent also the baroreceptor reflex modulation of the vascular bed It is here of interest that the carotid sinus reflexes may be inhibited also from the inferior olive (Smith and Nathan 1966) which is known to have intimate cerebellar connections.

As to the adrenergic cardiovascular control it is *a priori* possible that the reason for this differentiated interaction between the fastigial pressor response and the

baroreceptor reflexes is simply a result of the relative differences in effector responses to given rates of fastigial stimulation Thus the cardiac accelerans response may reach a maximum already at 10—20 imp/s of fastigial stimulation, whereas the blood pressure and vascular responses become maximal first around 50 imp/s If now a baroreceptor activation exerts a fairly uniform damping influence on the sympatho-excitatory effects, induced by e g high frequency fastigial stimulation this may result in a profound baroreceptor reflex influence on the vessels, and hence also on the blood pressure On the other hand, the reflex modulation of the accelerans discharge would have little or no effect on the myocardium as this is already driven at rates of sympathetic discharge that are well on the plateau of the hyperbolic frequency-response curve for the cardiac neuro-effector unit This difference between heart and vessels as to their responses to different rates of fastigial stimulation may, in turn, simply reflect the differences that are known to exist for these two adrenergic neuro-effector systems, thus the frequency response curves for the heart the spleen or the capacitance vessels with respect to direct sympathetic stimulation are far more hyper-bolic than those of the resistance vessels (see e g Mellander 1960) It appears, how-ever that the differentiated interaction between the fastigial pressor response and the baroreceptor reflexes involves also a centrally located element that particularly affects the reflex cardiac control, since the baroreceptor activation of the vagal heart supply was particularly strongly suppressed by fastigial stimulation

A similar type of differentiated interaction between baroreceptor reflexes and suprabulbar excitatory influences which can partly be ascribed to a central mechanism, has earlier been described for the hypothalamically induced defence reaction (Djojosugito et al 1970) In this case the baroreceptor impact upon the heart is to a considerable extent suppressed while that on the resistance vessels re-mains almost the same, with the net result that the heart delivers an increased out put at a relatively lower energy expense during the defence reaction, particularly favouring the blood supply to the muscles (Kylstra and Lisander 1970)

It is likely that more examples of such differentiated interaction between auto-nomic response patterns will be found as it appears to reflect inherent properties not only of the various neuroeffector systems in the periphery but also of the organization of central neuron pools governing the autonomic nervous system In fact findings in exersising man are also compatible with a more or less profound extinction of the baroreceptor reflex action on the heart (see Freyschuss 1970) Certainly the in-fluence of anesthesia in the present experiments cannot be responsible for the dif-ferences observed between cardiac and vascular neurogenic control although "resting" heart rate is considerably higher in anesthetized operated animals than in unanesthetized resting cats (often only 100—150 beats/min, Lisander, unpublished observation)

Miura and Reis (1969) found that the fastigial pressor response is abolished by destruction of the paramedian reticular nucleus, which has been reported to receive a heavy projection from the baroreceptor nerves (Miura and Reis 1968 Crill and Reis 1968) A possible interpretation is that the fastigial pressor response is brough

about by a mere suppression of baroreceptor relay stations On the other hand, a blood pressure rise can still be induced by fastigial stimulation when all baroreceptor stations are denervated (Achari and Downman 1970) The possibility remains, however, that the neuron pools of the medullary depressor area maintain some activity even when deprived of the baroreceptor impact The responses of the resistance vessels to fastigial stimulation in the present experiments were usually augmented if carried out with a background of enhanced sympathetic tone This mutual facilitation between the fastigial pressor response and the activity emanating from the bulbar 'vasomotor centre' when relieved of the baroreceptor inhibitory influences does not however, necessarily mean that the fastigial neurons exert their effects directly *via* the vasomotor centre, the finding may equally well reflect a convergence on *e g* the preganglionic neurons in the spinal cord

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Influence of Synthesis and Membrane Pump Inhibition on the Nerve Impulse Induced Disappearance of Noradrenaline from Rat Salivary Glands

By

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Abstract

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The cervical sympathetic trunk of rats was unilaterally stimulated with 5 imp/s for different time intervals up to 3 h and the contraction response of the lower eye lid was recorded At the end of the stimulation the [illegible] previously administered dl ^{3}H NA was meas[illegible] inhibition of the NA synthesis 500 mg/kg [illegible] was given to one group of rats and for bloc[illegible] protriptyline (initially 10 mg/kg) was administered to some other rats Also some rats received both drugs In un[illegible] ve stimulation while [illegible] tween tritiated and e[illegible] reduced after synthe[illegible] eriment in the untrea[illegible] ransmitter decrease [illegible] reduced transmitter output per stimulus and hence in a reduced effector response The retarded disappearance of ^{3}H NA seen after protriptyline treatment could also be explained by a reduced output per stimulus but other possibilities may also exist

Exogenously administered labelled noradrenaline (NA) is taken up and retained in the adrenergic nerve terminals of sympathetically innervated organs (Hertting *et al* 1961, Wolfe *et al* 1962), and can be released by electrical stimulation of the nerves (Hertting and Axelrod 1961) or by the physiological impulse flow (Hertting Potter and Axelrod 1962) The endogenous transmitter content of the nerves is also influenced by the impulse flow Although effector responses seem to be maintained during prolonged electrical stimulation (Orias 1932, Dye 1935) there is a marked decrease of the NA levels (Kernell and Sedvall 1964 Fredholm and Sedvall 1966) In some organs the NA content has been shown to increase when the normal ne[illegible] impulse flow is broken by decentralization (preganglionic denervation) or

ganglionic blocking agent (Karki, Paasonen and Vanhakartano 1959, Hertting Potter and Axelrod 1962, Sedvall 1964) Apparently the stores of sympathetic transmitter are influenced by the nerve impulse flow

Several processes may participate in the regulation of the transmitter release by nerve impulses The output of NA per stimulus is dependent on the frequency of the impulse discharge, often decreasing at high frequencies (Brown and Gillespie 1957, Haefely, Hurlimann and Thoenen 1965, Haggendal *et al* 1970) The synthesis of NA may also vary with the impulse activity of the neurons (*e g* Roth, Stjarne and v Euler 1966, Sedvall and Kopin 1967) Perhaps also other intraneuronal processes (*e g* storage function and metabolism of NA) might influence the extent of transmitter release The operation of local reflex mechanisms regulating the transmitter output per stimulus has been suggested by Haggendal (1969, 1970) The inactivation of the released transmitter can also affect the net release (Rosell, Kopin and Axelrod 1963, Langer 1970) Apart from the reuptake at the nerve cell membrane, "the membrane pump", which under physiological conditions probably accounts for the major part of the inactivation (Folkow, Haggendal and Lisander 1968), also extraneuronal accumulation and catabolism may participate (Folkow, 1952 Gillespie and Kirpekar 1965, Axelrod 1966, see also Iversen 1967) Finally, variations in blood flow can markedly influence the extent of local accumulation and reuptake into the nerve endings (Rosell Kopin and Axelrod 1963, Folkow, Haggendal and Lisander 1968)

In the present study the disappearance, following continuous sympathetic stimulation of the endogenous transmitter and previously accumulated ^3H NA from rat salivary glands was studied The aim of the investigation was to determine the quantitative relationship between the release and the level of transmitter In order to get an insight into the mechanisms involved the effects of NA synthesis inhibition and blockade of the membrane pump were also studied

Methods

Male Sprague Dawley rats weighing about 200 g were used Before the experiment the rats were placed in a room with a constant temperature of [illegible] hypothermia
dl Noradrenaline 7 ^3H (specific activity 6 6—9 7 C/r [illegible]
in a dose of 1 μg/kg was injected in a tail vein 3— [illegible]
rats were anesthesized with urethane about 1 g/kg [illegible] hetic trunk [illegible] t the level of [illegible] On the left [illegible] ical ganglion [illegible] dure the rat [illegible] on a bipolar [illegible] ement *trans* [illegible] the applied [illegible] d mainly to [illegible] tested to be [illegible] vered by a
supramaximal (usually [illegible] v 1—2 ms) at a frequency of [illegible] Grass S4 stimulator The stimulation was continued for a time period of 5—180 min

In one series of rats the stimulation was performed without any pretreatment of the rats In another series, 500 mg/kg of DL α methyl p tyrosine methylester (H 44/68) was given i p

1 h before the beg[illegible] chl[illegible] tha[illegible] tre[illegible] was performed with the same doses and at the same time intervals as above

Immediately after the stimulation was terminated the submaxillary plus sublingual glands of both sides were taken out, weighed and homogenized in ice-cold 0 4 N perchloric acid The NA fraction was isolated by [illegible] were divided int[illegible] tion counting af[illegible] 1963) In the ot[illegible] son and Rosengr[illegible]

In some contr[illegible] H44/68 treated rats was more carefully measured To these rats ^{3}H NA was not administered The response obtained in the beginning of the stimulation with 5 imp/s was measured After 1 h (18 000 [illegible] base lin[illegible] 4 000 impulses) stimulation was interrupted to conti[illegible] stimulation levels and thereafter the response

The [illegible] and an[illegible] mainly based upon an analysis of regression [illegible] strength of linear association was tested according [illegible] (1963) Some of the statistical values are presented in Table II In other cases where statistical comparisons are made the Student's t test was used

Results

Levels of tritiated and endogenous NA in unstimulated glands

For two reasons it was found most suitable to express the values of ^{3}H NA and endogenous NA found in the stimulated glands as a percentage of the corresponding content in the contralateral, unstimulated gland Firstly, the electrical stimulation was not always begun at the same time interval after the administration of ^{3}H NA, but for technical reasons this interval varied between 3 and 6 h Secondly, other losses of ^{3}H NA or endogenous NA than due to the nerve impulse flow could occur When expressing the data as a percentage of the unstimulated gland such losses would not influence the disappearance curves The values at zero number of impulses given in Fig 1—4 were obtained from rats treated exactly in the same way as the other rats of that series, but sacrified 3 h after the administration of ^{3}H NA and not subjected to stimulation There was no significant difference between the amounts of ^{3}H NA obtained in these unstimulated glands in the different series (untreated $0\,69 \pm 0\,16$ ng/g $n=4$, H 44/68 $1\,06 \pm 0\,26$ ng/g $n=5$ protriptyline $0\,74 \pm 0\,10$ ng/g, $n=4$ and H 44/68+protriptyline $1\,08 \pm 0\,04$ ng/g $n=4$) When the 2 groups which had not received H 44/68 (with or without protriptyline) were pooled ($n=8$) and compared with those 2 groups receiving H 44/68 which were likewise pooled ($n=9$), a statistical significant difference was found ($0\,72 \pm 0\,088$ and $1\,07 \pm 0\,357$, respectively $P < 0\,05$) For a discussion of an analogous phenomenon, observed in experiments using ^{3}H metaraminol see Almgren (1971) No certain trend towards increase or decrease of the ^{3}H NA levels in the unstimulated glands could be observed during the period of stimulation (3 h) in any of the experimental series

TABLE I The content of noradrenaline (NA) found in the contralateral, decentralized, unstimu-

S E M Figures in brackets denote number of experiments

Time of stimulation (min)	Pretreament			
	No pretreatment NA μg/g	H 44/68 NA μg/g	Protriptyline NA μg/g	H 44/68 + + protriptyline NA μg/g
0	1 22 (4) ±0 158	1 64 (2) ±0 110	—	—
15	1 12 (5) ±0 099	1 35 (4) ±0 178	1 13 (3) ±0 251	0 75 (2) ±0 040
120	1 68 (2) ±0 255	0 99 (3) ±0 166	1 03 (5) ±0 145	0 81 (3) ±0 029

The endogenous NA content found in the unstimulated glands at some intervals during the experiment is given in Table I In no cases were there any significant changes in NA levels, but a tendency to increase in the untreated rats and to decrease in the H 44/68-treated rats can be noted Also, no significant difference was found between the NA contents of the unstimulated glands in the various groups 15 min after the beginning of the stimulation

Stimulation-induced disappearance of tritiated and endogenous NA from salivary glands of untreated rats

The disappearance of ^{3}H NA from the salivary gland at nerve stimulation closely followed a single exponential decay (Fig 1) A reduction to 50 per cent was reached after 14,300 impulses or about 48 min with an impulse flow of 5 /s When 54,000 impulses were given during 3 h only small amounts of labelled amine remained in the stimulated gland, around 0 05 ng/g The endogenous NA level at that time amounted to some 0 3 μg/g Also the disappearance of the endogenous NA closely followed a single exponential course (Fig 1), which was, however considerably slower than that of the labelled amine (Table II)

Stimulation-induced disappearance of tritiated and endogenous NA from salivary glands of H 44/68-treated rats

H 44/68, 500 mg/kg was given i p to the rats 1 h before the stimulation was started In the stimulated gland the disappearance of both labelled and endogenous NA seemed to follow single exponential functions, of about the same half-lives (Fig 2, Table II) Endogenous NA in the H 44/68 treated rats disappeared faster following stimulation than the endogenous NA of untreated rats ($P<0\ 001$) The decrease of labelled NA in the rats treated with H 44/68 did not significantly differ from that of untreated rats

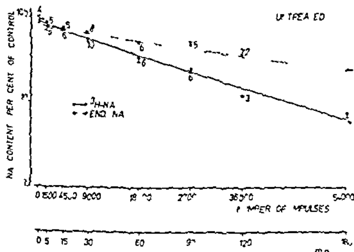


Fig 1 The levels of tritiated and endogenous noradrenaline (NA) found in rat salivary glands at various time intervals during continuous stimulation of the right cervical sympathetic chain with 5 imp per second 1 μg/kg ^{3}H NA was administered i v 3—6 hrs before the stimulation was started The values are expressed as a percentage of the corresponding contents found in the contralateral decentralized gland and given as mean ± S E M The figures represent the number of experiments When less than 3 experiments were performed the individual (percentual) values are given The lines were drawn according to the statistical calculations (see Table II)

TABLE II Statistical evaluation of the material presented in Fig 1—4 and comparison between the slopes The correlation coefficients were significant within $P < 0\ 01$ for all groups The F values of linear association were significant within $P < 0\ 01$ in all cases although in the last group (h) very near the limit for significance End NA denotes endogenous noradrenaline n.s = $P > 0\ 05$

	Untreated		H 44 68		Protriptyline		H 44/68 + Protriptyline	
	^{3}H NA	End NA	^{3}H NA	End NA	^{3}H NA	End NA	^{3}H NA	End NA
Correlation coeff	—0 96	—0 88	—0 93	—0 78	—0 85	—0 62	—0 81	—0 53
Regression coeff	—0 0063	—0 0029	—0 0073	—0 0093	—0 0045	—0 0023	—0 0056	—0 0075
50 per cent reduction reached at (no of imp)	14 300	31 000	19 300	9 700	20 100	38 800	16 100	12 000
time (min)	47 5^a	103 3^b	41 0^c	32 2^d	66 9^e	129 4^f	53 7^g	40 1^h
Strength of linear association F value	462	125	241	48 0	99 2	23 8	73 2	9 47
Number of exp	44	40	43	33	40	31	40	26

	P		P		P		P
a—b	< 0 001	a—c	n s	e—g	n s	b—h	< 0 025
c—d	n.s	a—e	< 0 005	a—d	< 0 025	d—h	n.s
e—f	< 0 005	a—g	n s	b—d	< 0 001	f—h	< 0 025
g—h	n s	c—g	< 0 05	b—f	n.s		

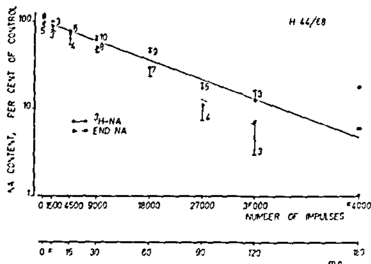


Fig 2 Disappearance of endogenous and tritiated noradrenaline from rat salivary glands by nerve stimulation, as in Fig 1 Rats pretreated with DL-α methyl p tyrosine methylester (H 44/68), 500 mg/kg i p 1 hr before the beginning of the stimulation

Stimulation induced disappearance of tritiated and endogenous NA from salivary glands of protriptyline treated rats

10 mg/kg of protriptyline was given i p to the rats 15 min before the stimulation was started Additional doses were given after 1 and 2 h of stimulation With this treatment there still seemed to be an exponential decline of ^{3}H-NA by stimulation 50 per cent reduction was reached after about 20 000 impulses or 67 min of stimulation

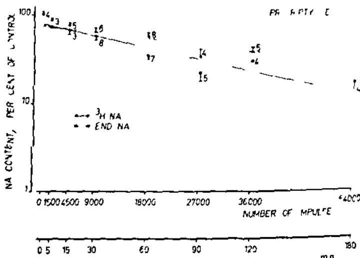


Fig 3 Disappearance of endogenous and tritiated noradrenaline from rat salivary glands by nerve stimulation as in Fig 1 Rats pretreated with protriptyline chloride, 10 mg/kg i p 15 min before the beginning of the stimulation When the experiments were continued for more than 1 hr an additional dose of 5 mg/kg i p was given every hour

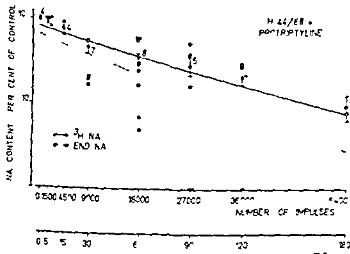


Fig 4 Disappearance of endogenous and tritiated noradrenaline from rat salivary glands by nerve stimulation, as in Fig 1 Rats pretreated with H 44/68 as in Fig 2 and protriptyline as in Fig 3 Due to the considerable variance of the endogenous NA levels these are given as individual (percentual) values

with 5 imp/s (Fig 3, Table II) The rate of disappearance compared to the untreated animals was retarded ($P < 0.005$) The retardation of the endogenous NA disappearance was, however, less pronounced compared to the untreated animals and proved not significant (Fig 3 Table II)

Stimulation induced disappearance of tritiated and endogenous NA from salivary glands of rats treated with H 44/68 and protriptyline

Also after the combined treatment of the animals with H 44/68 and protriptyline the disappearance of ^{3}H NA seemed to follow a single exponential course where 50 per cent reduction was reached after about 16 000 impulses or about 54 min of stimulation with 5 imp/s (Fig 4 Table II) This was not significantly slower than the disappearance of ^{3}H NA from the untreated or protriptyline treated rats by stimulation Compared to the rats treated with H 44/68 alone there was a marked retardation of the ^{3}H NA-disappearance induced by the combined treatment with protriptyline ($P < 0.05$) The reduction of the endogenous NA level by stimulation was more variable (Fig 4), and the strength of linear association considerably less than for the other groups (Table II) The half life was not significantly prolonged after treatment with both drugs compared to after H 44/68 treatment alone

Contraction response of the lower eye lid to continuous stimulation with 5 imp per second

The main purpose of studying the contraction response of the lower eye lid was detect possible changes of the response during the period of stimulation No were made to compare the responses of different rats since a certain v

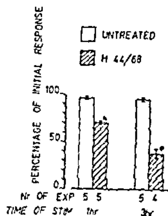


Fig 5 The contraction response of the lower eye-lid to stimulation [illegible]
second after [illegible]
of stimulatic[illegible]
pretreated [illegible]
44/68) 500 mg/kg 1 hr before the start of the stimulation The other rats were not pretreated The values are presented as a percentage of the contraction response at the beginning of the stimulation * — *differs from untreated at* $P < 0.001$

responses recorded could be due to slight variations in the registration technique It would be stated, though, that no certain differences in initial responses were observed after the different pretreatments used

In the untreated rats the contraction response remained essentially unchanged during the whole stimulation period studied (Fig 5) After pretreatment with 44/68 a gradual reduction of the contraction response was recorded to some 40 cent of the initial response after 3 h of stimulation (Fig 5)

Also in the protriptyline treated animals there seemed to be an essentially unchanged contraction of the eye-lid throughout the 3 h stimulation period The relaxation time on the other hand was considerably prolonged When in some other rats protriptyline was given after the beginning of the stimulation a small and short lasting increase of the contraction response was observed about 5 min after the administration of the drug

After treatment with both H 44/68 and protriptyline there was a more variable reduction of the contraction response of the eye lid than after H 44/68 alone In most cases however the reduction was more pronounced than after H 44/68 only, reaching as a mean about 50 per cent reduction after 60 min of stimulation, but the response never completely disappeared during the 3 h of stimulation studied

Discussion

The present investigation was performed to study the release of the adrenergic transmitter from peripheral sympathetic nerve endings In most earlier studies dealing with these problems the overflow of transmitter into blood or perfusate following nerve stimulation has been measured It was thus considered of interest to attack the problems along another line namely by measuring the amounts of transmitter lost from a sympathetically innervated organ at different time intervals following nerve stimulation at a constant frequency Unphysiological manipulations could be reduced to a minimum and the constant impulse flow kept within the physiological range (Folkow 1952) Also it was possible to study the transmitter release during a relatively long period of sympathetic activity and calculate its relation to the stores

of transmitter By the use of relatively specific drugs for inhibition of the NA synthesis and blockade of the reuptake mechanisms it was hoped that a quantitation of these two mechanisms would be possible

During the time period studied the decrease of the labelled as well as of the endogenous NA levels closely followed single exponential functions As could be expected in this case, with the synthesis of new NA going on the tritiated NA was found to decrease at a considerably higher rate than did endogenous NA The data could indicate that the amount of transmitter liberated per impulse is proportional to the amount stored However, the influence in this case of the NA synthesis, reported to increase in the rat submaxillary gland at sympathetic stimulation (Sedvall and Kopin 1967), is hard to evaluate

In order to avoid difficulties due to the NA synthesis α methyl p-tyrosine methyl ester (H 44/68) was used to inhibit the first step of this synthesis (Nagatsu Levitt and Udenfriend 1964, Spector, Sjoerdsma and Udenfriend 1965 Corrodi and Hanson 1966) This treatment greatly increased the disappearance of endogenous NA from the stimulated glands so that there was no longer any significant difference between the declines of endogenous and labelled NA In other words the specific activity remained constant during the experiment As the disappearance of both labelled and endogenous transmitter was exponential also after synthesis inhibition it follows that the net release per impulse i e after subtraction of the amount recaptured by the membrane pump in this case is proportional to the amount of transmitter in the total store

For studying the effect of reuptake inhibition protriptyline was used as it is a very effective inhibitor of amine uptake in peripheral adrenergic nerves (Carlsson and Waldeck 1965 a) A dose of 10 mg/kg is necessary to obtain nearly maximal inhibition of ^{3}H NA uptake into the rat submaxillary gland (Almgren and Jonason 1971) The results obtained in the present study a markedly delayed disappearance of ^{3}H NA and a (not significant) tendency to retardation of the disappearance of endogenous NA, can at present not be fully explained Similar observations have been made using other, structurally related membrane pump blockers (Carlson and Waldeck 1965 b, Titus *et al* 1966 Folkow Haggendal and Lisander 1968 Haggendal *et al* 1970), and different explanations have been suggested A direct effect of the drug itself on the release or redistribution mechanisms can not be excluded However, also in this case the disappearance of both labelled and endogenous NA followed single exponential decays Thus it seems probable that also the total release of transmitter i e including the part normally recaptured by the membrane pump is proportional to the store of transmitter The exponential decay of tritiated NA following nerve stimulation in the rats treated with both H 44/68 and protriptyline supports this assumption

A calculation based on the half life of endogenous NA in the stimulated gland after synthesis inhibition reveals that about 5×10^{-5} of the amount of NA stored in the gland disappears per impulse Assuming that at least 50 per cent of the NA released per impulse will be recaptured by the nerve terminals (Folkow Haggendal a

sander 1968) the release per impulse will be at least 10^{-4} of the NA store As will be discussed below this figure may be even higher when the NA synthesis is intact

After synthesis inhibition there was a marked decline of the contraction response of the lower eye-lid, not seen in the untreated animals (Fig 5) Although the effector response was not measured in the same organ as the amine levels it might still serve as an indicator of nerve function in the salivary glands, since the sympathetic nerves of both organs originate in the superior cervical ganglion and transmitted the same number of impulses

Taken together, the data from the present study seem to be best interpreted in terms of a compartmentalization of the transmitter in a small, functional pool, where the transmitter is immediately available for release, and a bigger 'less available' storage pool (Trendelenburg 1961, *cf* also Carlsson 1965, Iversen 1967) Under the present experimental conditions the tritiated NA seems to be mainly localized within the "less available" storage pool, since after synthesis inhibition tritiated and endogenous NA disappeared at about equal rates In order to be released by the nerve impulses ^{3}H-NA must then be transferred to the "available" pool

Three major mechanisms can participate in the refilling of the "available' pool 1) recapture of released transmitter, 2) synthesis of new transmitter, and 3) redistribution of the transmitter stored in the "less available' pool After inhibition of NA synthesis all the transmitter (both tritiated and endogenous) lost following nerve stimulation must emanate from the storage pool The rate of transmitter disappearance (or net release) in this case must be the same as the rate of redistribution from the 'less available" to the "available" pool Assuming that the part recaptured by the membrane pump during this experiment was a constant fraction of the amount transmitter released it follows that the output per stimulus, after synthesis inhibition, continuously decreases Under the same assumption this may be the case also with an intact NA synthesis during constant stimulation with 5 imp/s, as there was a gradual decrease of the endogenous NA levels also in these experiments However the level of endogenous NA in the glands of untreated rats after 3 h of stimulation was, if anything, lower than the level found in the H 44/68 treated rats after 1 h of stimulation (24 and 30 per cent respectively) and yet the contraction response of the lower eye lid was essentially unchanged in the untreated rats while it was reduced to about 70 per cent in the H 44/68-treated animals at the corresponding time intervals It might thus be concluded that at about equal reductions of the transmitter content the release per stimulus is bigger in animals with an intact NA synthesis than in animals where this synthesis is blocked This result indicate that the synthesis is necessary to keep the transmitter content of the "available" pool at a normal level Such a concept will be in accordance with the reports of a preferential release of newly synthesized transmitter (Kopin *et al* 1968, Stjarne and Wennmalm 1970) It could also provide an explanation for the more rapid decrease in function than in amine levels after synthesis inhibition, observed in the central nervous system, where the turnover and thus probably the impulse flow is high (Corrodi and Hanson 1966, Svensson and Waldeck 1971)

Changes in blood flow may greatly influence the disappearance of NA in animals with an intact recapture mechanism (Rosell, Kopin and Axelrod 1963) but not after blockade of the membrane pump (Folkow, Haggendal and Lisander 1968 It can not be excluded that the magnitude of the NA reuptake in the present experiments increased due to a decrease of the blood flow in the stimulated glands when pro triptyline was not administered It is, however, uncertain if a vasoconstrictor response was maintained during the whole stimulation period (*cf* Burgen and Emmelin 1961)

The great variance of the endogenous NA values in the rats treated with both H 44/68 and protriptyline precludes any certain conclusions to be drawn from those data Also the reduction of the effector response was more variable than after H 44/68 alone, but tended to be somewhat greater The tendency to a retarded disappearance compared to H 44/68 alone of both tritiated and endogenous NA is in line with the corresponding difference in animals not treated with H 44/68

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The Effect of Stomach Distension on the Efferent Activity in the Chorda Tympani Nerve of the Rat

By

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Abstract

Hellekant, G. *The effect of stomach distension on the efferent activity in the chorda tympani nerve of the rat.* Acta physiol. scand. 1971. 83. 527—531.

The efferent activity in the chorda tympani nerve of the rat was recorded under neuroleptic analgesia. The results show that this activity decreased when the stomach was distended. The effects caused originated from the stomach and diminished after cutting the vagal nerves. It is suggested that the efferent activity of the chorda tympani nerve can be influenced by hunger and satiety.

Efferent impulses in the chorda tympani nerve of rats were reported in an earlier study (Hellekant 1971 c). In that study three different functions were suggested for these impulses. One was related to hunger and thirst. In the attempt to investigate this possibility a method was looked for which would switch the rat from a stage of hunger to one of satiety. Distension of the stomach seemed to offer such a method because it is known that it elicits a sensation of satiety and produces a loss of appetite (Herrin and Meek 1933). The present study was therefore made with the aim of recording efferent impulses in the chorda tympani nerve during moderate distension of the stomach of the rat.

Methods

Adult Sprague Dawley rats were usec [illegible] ere deprived of food for 18–24 h bef re the expt. They were anesthetized w [illegible] previous study (Hellekant 1971 c) [illegible] muscular artifacts. The animals wer [illegible]

One chorda tympani nerve was dissected out and cut peripherally. The activity of the nerve was [illegible]

[illegible] sion in the wall in the pyloric region. The incision was then sutured. The balloon was c[illegible] nected to a 20 ml syringe outside the abdomen. A similar balloon was in some cases outside and along the stomach. The other method demanded more surgery but [illegible]

because the balloon was sometimes more difficult to empty than the stomach Three polyethylene tubings of different diameters were used One was inserted into the stomach through an incision in the oesophagus at the level of the larynx Its diameter was chosen so that it occluded the lumen of the oesophagus It served as inlet to the stomach The second tubing was the smallest one It was inserted into the stomach through the wall which was then sutured It served as an intragastric pressure recording device The third tubing had the largest diameter It was inserted into the stomach through an incision close to the pylores and served as an outlet from the stomach The stomach was distended with Ringer's solution or tap water at body temperature

Results

Fig 1 shows simultaneous recordings of the summated efferent activity in the chorda tympani nerve, upper trace, and the systemic blood pressure, middle trace, during two distensions of the stomach, lower trace The stomach was filled with Ringer's solution until an intragastric pressure of 10 mm Hg was recorded Fig 1 also shows that the efferent activity of the chorda tympani nerve decreased during these periods Little effect on the blood pressure was recorded However, this was not always the case Several animals displayed a considerable increase in blood pressure during the distension This raised the question whether the decrease of the neural activity observed was an effect of the stomach distension or of the increased intraabdominal pressure

An attempt to solve this problem is shown in Fig 2 The efferent activity in the chorda tympani nerve, expressed in imp/s, has been plotted in the upper part of the graph The middle curve displays the blood pressure, while the lower one indicates the intragastric pressure Two consecutive recordings were plotted First the stomach was filled with 20 ml Ringer's solution This increased the intragastric pressure to about 15 mm Hg and decreased the efferent activity in the chorda tympani nerve by about 33 % The same volume was then injected at the same rate into an extragastric balloon The graph shows that the blood pressure rose in a manner similar to when the stomach was filled but that the efferent neural activity did not decrease Fig 2 suggests that this effect on the neural activity was caused by the distension of the stomach Increase of intraabdominal pressure in itself caused no change in the efferent chorda tympani nerve activity

It is known that the impulses from the gastric receptors responding to distension are mainly mediated through the vagal nerves (cf Sharma 1967) Therefore, the

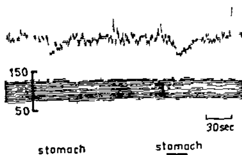


Fig 1 The record shows from the top the summated efferent activity of the chorda tympani nerve the arterial blood pressure and a signal which indicates the filling of the stomach with Ringer's solution The record shows a decrease in neural activity during the stomach distensions

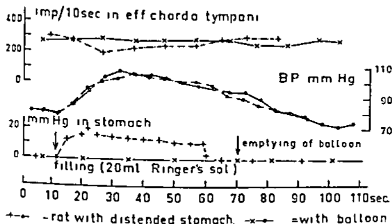


Fig 2 The graph shows the effect of intragastric and extragastric distension on the efferent chorda tympani nerve activity and the blood pressure The graph shows that no decrease of efferent activity was recorded during the extragastric distension

effect of cutting the vagal nerves was studied Fig 3 presents the result of such an experiment The efferent impulse activity of the chorda tympani nerve is plotted in the upper part of the graph The circles represent this activity expressed in imp/s The average activity, measured over 10 s is also shown (crosses) The middle part of the graph displays the blood pressure and the lower one the intragastric pressure The arrows mark the onset of flow into the stomach The left hand graph was obtained before, the right hand after the vagal nerves were cut Fig 3 shows that transection of the vagal nerves reduced the effect of stomach distension on the efferent chorda tympani activity In this experiment the decrease of efferent activity was 35 % before and 20 % after the vagotomy

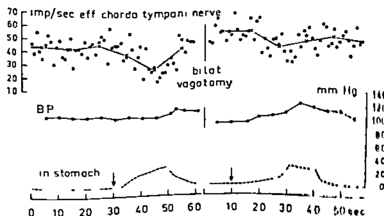


Fig 3 The graph shows the efferent chorda tympani activity [illegible] and intragastric pressure before and after cutting the vagal nerves The graph indicates a difference [illegible] pression of activity caused after this cutting

In summary, the results presented show that distension of the stomach in the rat may depress the efferent activity of the chorda tympani nerve, that these effects emanate from the stomach and that the depression caused diminishes after cutting the vagal nerves

Discussion

The results presented show that distension of the stomach may affect the efferent activity in the chorda tympani nerve This discussion will present some earlier observations which make such a relation possible

A number of studies indicate that stimulation of the stomach can influence the activity of the salivary glands on the taste receptors Thus Bulygin (1962) showed that distension of the stomach inhibits salivation in the dog In man an increased taste threshold from single fungiform papilla has been demonstrated after feeding through a gastric fistula (Zaiko and Lokshina 1963) Decreased activity in the afferent fibres of the glossopharyngeal nerve of the frog and toad during stomach distension has been reported by Esakov (1961) and Brush and Halpern (1970)

The number of studies which indicate that the chorda tympani nerve mediates this influence is less The existence of such an influence on the salivary glands seem unquestionable though no recordings of these impulses have been published (Emmelin 1967 p 595) With regard to the taste receptors there is some indication of such an influence (Hellekant 1970 1971 a b c)

The link between the CNS and the stomach is better known Activity of single neurons in the hypothalamic feeding centres has been recorded (Anand and Pillai 1967) during gastric distension The impulses travel in the vagal and splanchnic nerves the vagal being more important (Lalich Meek and Herrin 1936 Nijima 1970) Studies by Paintal Iggo and others have clarified some of the characteristics of the receptors which elicit these impulses (Paintal 1963 Iggo 1966 There are two types that can be considered in the present context They are both situated in the walls of the stomach one in the muscular coat the other in the serosa They are mechanoreceptors and show certain characteristics which may explain some of the observations in the present study They adapt slowly during stimulation Paintal 1954 Iggo 1957 This may explain the slight return of activity to the prestimulation level illustrated in Fig 2 before the stomach was emptied During distension impulses travel in the splanchnic and the vagal nerves causing a reflex inhibition of gastric tonus (Lalich *et al* 1936 Iggo 1966 This reflex is considerably disturbed if the vagal nerves are cut *cf* Sharma 1967 This may explain the difference between the rises of the intragastric pressure in Fig 3 a difference which is otherwise difficult to explain because the flow rate was the same Fig 3 shows also that cutting the vagal nerves diminished but did not abolish the effect of stomach distension on the efferent chorda tympani activity This may be explained by the observation that the splanchnic nerves also mediate impulses from the gastric receptors (Sharma 1967)

In summary, a number of studies have described the characteristics of the gastric distension receptors outlined their connections with the CNS and shown the effects of stomach distension on the neurons of the hypothalamic feeding centres It seems reasonable to conclude that the changes in the efferent activity of the chorda tympani nerve observed here were caused by stimulation of such gastric distension receptors Further some of their characteristics seem to be reflected in features of the chorda tympani activity The present study also demonstrates that changes in this activity can be caused by a stimulus which is known to influence hunger and thirst

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Acta physiol scand. 1971. 83 532—541

From the Department of Physiology, University of Goteborg Sweden

The Hemodynamic Consequences of Regional Hypotension in Spontaneously Hypertensive and Normotensive Rats

By

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Abstract

FOLKOW, B M GUREVICH M HALLBÄCK, Y LUNDGREN and L WEISS *The hemodynamic consequences of regional hypotension in spontaneously hypertensive and normotensive rats* Acta physiol scand 1971 *83* 532—541

In 3 week old spontaneously hypertensive rats (SHR) and normotensive control rats (NCR) the aorta was ligated distally to the renal arteries lowering the blood pressure in the hind quarters some 30—50 per cent compared with ordinary NCR or SHR 6—10 weeks later the hindquarters of an ordinary NCR were perfused simultaneously with those of an aorta ligated NCR or SHR in 20 paired experiments at constant flow with an oxygenated plasma substitute from maximal dilatation to maximal constriction as induced by graded noradrenaline (NA) doses Constructed resistance curves of the aorta ligated NCR and SHR hindquarters showed a 40—50 % decreased resistance at maximal dilatation with almost proportional decreases of the steepness of the dose response curve and of the maximal pressor response when compared with ordinary NCR while the threshold sensitivity to NA was the same as in unligated shamoperated NCR The difference from unligated SHR was still more striking yielding three separate sets of resistance curves each related to the pressure level present in the hindquarters during the months prior to the perfusion The results indicate an adaptive structural change of the resistance vessels in the chronically hypotensive hindquarters with a media reduction and considerably increased vascular lumina Thus the elimination of the pressure load seems capable of completely preventing the development of the adaptive media increase that otherwise seems to occur in SHR An altered pressure load decreased or increased evidently affects the design of the resistance vessels rapidly and this in turn will greatly contribute to the hemodynamic differences characterizing hypo- and hypertension as compared to normotension

Several hemodynamic studies of regional vascular beds in man suggest that the increased resistance in essential hypertension may to a great extent be caused by a structural adaptation of the systemic resistance vessels This adaptation in response to the increased pressure load leads to an increased wall/lumen ratio, presumably as a result of mainly media hypertrophy which tends to raise the resistance even

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during maximal dilatation by encroaching upon the lumen (Folkow Grimby and Thulesius 1958 Conway 1963) Folkow (1956) and Sivertson (1970 illustrated how such a type of structural change would by its mere presence also lead to exaggerated luminal narrowing of the resistance vessels for given degrees of smooth muscle shortening i e increasing vascular reactivity without necessitating any altered smooth muscle reactivity The reverse would be the case at decreases of the wall/lumen ratio Morphological studies of the resistance vessels particularly convincingly the investigation performed by Furuyama (1962) have revealed a considerable media hypertrophy in human hypertension especially affecting the larger resistance vessels

Recent experiments by Folkow *et al* (1970 a b) on the spontaneously hypertensive rat (SHR see Okamoto 1969) have confirmed and extended the above mentioned findings in man Thus the entire systemic vascular bed as well as the isolated hindquarters of these hypertensive rats exhibit a raised flow resistance even during maximal dilatation compared to normotensive control rats (NCR) Further graded noradrenaline (NA) infusions to isolated NCR SHR hindquarters showed identical smooth muscle sensitivity of the hindquarter vessels as judged by the NA threshold , but an increased steepness of the SHR curve relating dosage to the resistance response and also an increased maximal pressor response i e an enhanced contractile strength of the SHR vessels All these characteristics are to be expected if the resistance vessels of SHR and NCR differ mainly or only in terms of the bulk of contractile tissue present in their walls and if this structural difference is largely proportional to the difference in resting blood pressure in SHR and NCR

In order to study in which way and how rapidly resistance vessels adapt in structure and consequently in reactivity as a result of a sustained shift in blood pressure level Folkow and Sivertson (1968) produced regional hypotension in cats by occluding suitable arterial branches in one of the hindlimbs Already after 3—5 weeks the resistance vessels of the corresponding calf region displayed considerably reduced pressor responses to suprathreshold NA amounts or to constrictor fibre activations resulting in a less steep resistance curve than in the normotensive control limb while the NA sensitivity was largely unchanged as judged from measurements of the NA threshold These results strongly suggest a rapid structural adaptation of the resistance vessels and in such a direction that their wall/lumen ratio becomes reduced as a result of even brief periods of hypotension This was clear also from morphological studies of corresponding arterial branches in the two limbs

As thus the structurally determined wall/lumen ratio of the resistance vessels in normotensive cats evidently adjusts readily to changes in the regional pressure load an exploration was made whether and to what an extent the SHR resistance vessels respond in the same general way For such purposes hindquarter preparations of SHR and NCR which had been artificially kept hypotensive for varying time periods were compared to ordinary SHR and NCR hindquarter preparations with respect to the hemodynamics of their vascular beds from the level of maximal dilatation up to that of maximal constriction

Methods

In 10 three week old spontaneously hypertensive rats (SHR) and 10 normotensive matched control rats (NCR) the aorta was ligated during ether anesthesia half a cm distally to the renal arteries To serve as controls for this operation a group of NCR, subsequently used for the paired perfusions together with unoperated NCR, were operated in a similar way with dissection of the abdominal aorta, though no ligature was placed around this vessel — Within few days these young animals recovered seemingly completely from the operation, evidently due to rapid collateral development Their growth was largely normal and their hindquarter weight was less than 5 per cent below that of the controls

6—16 weeks later 20 paired experiments were performed on the isolated hindquarters of the aorta ligated SHR-NCR, being then perfused in parallel to hindquarters of ordinary SHR (5) or NCR (15) at constant flow conditions while noradrenaline (NA) was infused from subthreshold to supramaximal doses, starting from the level of maximal dilatation The technique used in these paired perfusions, the recording of the resistance changes and the construction of the "resistance curves", characterizing the individual vascular beds, has been described earlier in detail (Folkow *et al* 1970 b) Briefly, after having first estimated the arterial pressure in the hindquarters of the anesthetized animals by a catheter inserted towards the aortic bifurcation *via the tail artery, each pair of experimental animals was simultaneously eviscerated* and the abdominal aorta and caval vein were freed for about 2 cm just proximally to their iliac arborizations The animals were heparinized The perfusion of the hindquarters was started immediately when the distal ends of the abdominal aortas had been connected to the perfusion system The hindquarters were then isolated by standardized mass ligatures at identical levels The venous effluent was allowed to leave freely through the widely opened inferior caval vein As the tail artery in these preparations was already cannulated and used for direct recording of the perfusion pressure the tail was entirely excluded from the perfusion by a tight ligature and so were the feet by tight ankle ligatures leaving a preparation mainly consisting of the hindquarter muscles It was checked how much this exclusion of the tail and feet sections influenced the hindquarter resistance at maximal vasodilatation This obstruction which [illegible]ided nearly 10 % of the hindquarter tissue raised the resistance 15—20 % illustrating the low resistance of these predominantly cutaneous vascular beds, normally utilized for thermoregulating purposes

As in earlier studies (Folkow *et al* 1970 a b) oxygenated Tyrode solution at 38 C was used as perfusate but the colloid content (Ficoll a synthetic polymer of sucrose and epichlorohydrin m w about 80 000 kindly supplied by AB Pharmacia, Sweden) was kept at 4 % instead of at 3 % as in the earlier studies which raised the viscosity of the perfusate about 10 % and further reduced the tendency of oedema formation

After about 20—30 min of perfusion at identical and constant flows (usually 10 ml/min × 100 g) the perfusion pressures had stabilized at a low level Large doses of papaverine were injected to secure complete vascular relaxation When maximal dilatation had thus been established but after papaverin washout, NA dissolved in the perfusion medium was infused to both preparations in equal concentrations The NA doses could be increased in a stepwise fashion from subthreshold to supramaximal doses Definitely maximal pressor responses which in such a constant flow system reflect the maximal contractile strength of the vascular beds were achieved by adding slug injections of huge doses of NA followed by high concentrations of vasopressin (10 IE) and by barium ions (Ba^{++}) (30—50 mg)

Flow resistance at maximal dilatation and the resistance responses to NA and the other pressor drugs were calculated for each pair of hindquarter preparations and the resulting "resistance curves" were plotted with the log NA dose per ml perfusate on the abscissa and the pressor response on the ordinate For details concerning the characteristic key points of these "resistance curves" see Folkow *et al* (1970 b) From these "key points" characterizing the individual curves mean resistance curves representing the different animal groups were deduced and plotted

Results

The "resting" *carotid* blood pressure in the aorta-ligated SHR and NCR (2—6 months of age) was during nembutal anesthesia 180 ± 9 (SEM) and 135 ± 4 mm Hg, their tail artery pressures being 97 ± 12 and 80 ± 6 mm Hg respectively For the unligated SHR which were 6—8 months old, the mean carotid blood pressure

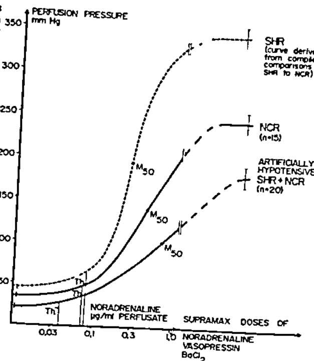


Fig 1 Average "resistance curves" of constant flow perfused hindquarter vascular beds from ordinary SHR and NCR and from aorta ligated SHR NCR Starting from maximal vasodilatation the curves show the relationship between increasing noradrenaline (NA) concentrations and the resulting resistance increases up to maximal pressor responses, which reflect the maximal contractile strength of the resistance vessels Note how the three curves differ in due proportion to the differences in "resting blood pressure in the SHR hindquarters (203 ± 8 mm Hg) in the NCR hindquarters (145 ± 4 mm Hg) and in the hypotensive hindquarters of the aorta ligated SHR-NCR (97 ± 12 and 80 ± 6 mm Hg here pooled into one curve) M_{50} denotes 50 per cent of the maximal pressor response and NA threshold sensitivity is denoted Th which for the aorta ligated SHR NCR curve is put within inverted commas since the "hypersensitivity is here only apparent and due to an artefact (see text)

was 203 ± 8 mm Hg and that of the unligated NCR was 145 ± 4 mm Hg tail artery pressures being here only a few mm Hg lower than the carotid pressures

In 20 paired experiments the isolated hindquarters of the aorta ligated NCR (10) and SHR (10) were perfused at constant flow about 10 ml/min × 100 g in parallel with those of unligated NCR (15) and SHR (5) starting from the level of maximal dilatation Noradrenaline (NA) was then infused from subthreshold to supramaximal amounts, and definitely maximal pressor responses were achieved as mentioned in Methods, after which resistance curves were constructed as also described earlier

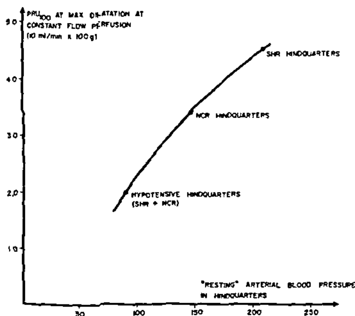
The relationships between the mean resistance curves representing a) unligated SHR (deduced from a large material of comparisons between NCR SHR partly shown in Folkow *et al* 1970 b) b) unligated NCR and c) aorta ligated SHR NCR (which were pooled because they differed fairly little both in resting hindquarter pressure and in shape of resistance curves) are illustrated in Fig 1 Here the following key points should be considered 1) Resistance at maximal dilatation 2) NA threshold' (defined as the NA concentration causing a 25 % increase of resistance from that at maximal dilatation) 3) Slope of the steep part of the resistance curve 4) Maximal pressor response and 5) 50 % of this maximal response (= M_{50} which should not be mistaken for ED_{50} since any difference as to wall/lumen ratio of resistance vessels will also greatly influence M_{50}, see Folkow *et al* 1970 b)

peculiarities in these resistance curves should first be briefly commented upon The NA 'threshold' appears to be lower in the aorta ligated animals as their resistance curve exhibits an earlier, but slight rise of its initial flat part This is, however, an "artefact" that is caused by a sympathetic denervation of a small fraction of the hindquarter vessels, inflicted by the earlier aortic isolation and ligation which damages some of the sympathetic supply This was clear from the fact that ordinary NCR, if earlier exposed to an identical aortic dissection but without ligation exhibited the same type of early slight rise in their resistance curves Therefore, these circumstances indicate that there was, in reality, no significant difference in NA sensitivity of the smooth muscles in the three groups of hindquarter vascular beds

It is on the other hand, clear from Fig 1 that flow resistance at maximal dilatation is some 40 % lower in the 'hypotensive' SHR-NCR hindquarters than in ordinary NCR which is again, some 35 % lower than that in ordinary SHR These differences are highly significant — Only in this particular respect the hypotensive SHR and NCR hindquarters differed significantly, and almost in proportion to the small difference in their tail artery pressures In other respects their resistance curves were so close together that it seemed rational to "pool" them into one average curve representing all the hypotensive vascular beds

Further, Fig 1 shows that this hypotensive resistance curve is far less steep than that of unligated NCR and, when compared with unligated SHR the difference is still more striking The slopes are best represented by the tangents of the fairly straight steep parts of the curves as compensated for the different initial resistances (for details see Folkow *et al* 1970 b), these tangents being for the hypotensive the NCR and the SHR resistance curves 1 9±0 2 2 6±0 3 and 4 0±0 4 respectively also these differences are highly significant There are correspondingly marked and highly significant differences concerning the maximal pressor responses reflecting the maximal contractile strength of the resistance vessels also M_{50} differs characteristicly being placed well to the right for the hypotensive resistance curve in relation to M_{50} for NCR and particularly for SHR As discussed earlier in detail (Folkow *et al* 1970 b) these characteristic differences and similarities between the resistance curves strongly suggest the presence of structural differences in wall/lumen ratio of the resistance vessels those of the hypotensive hindquarters being wider and more thinwalled and those of the SHR narrower and more thickwalled than the NCR ones

Concerning the changed wall thickness the media layer appears to be the crucial element to judge from the considerable differences in maximal contractile strength displayed by the resistance vessels in the three groups of animals However the aorta ligated animals differ even more from the ordinary NCR SHR with respect to average bore of the maximally relaxed resistance vessels This is most clearly evident when the resting blood pressure level of the three groups of animals is related separately to the resistance at maximal dilatation and to the maximal pressor response (Fig 2) It should be stressed however that the hindquarter blood pressure was measured during standardized anesthesia and this pressure is probably as



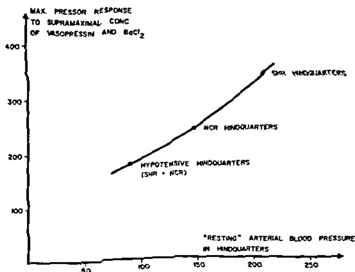


Fig 2 *Upp* ... ordinary SH... tation *Low*... strength of ...
Note that the aorta ligated SHR NCR differ relatively more from ordinary SHR NCR concerning resistance at maximal dilatation than in maximal contractile strength, for... discussed in text

an average lower in the aorta ligated rats when they are awake and normally active The reason is that the consequently higher blood flow rates to their hindquarters will imply a bigger pressure drop along the collaterals overbridging the aortic occlusion and hence a lower average level of regional arterial pressure This must be especially true for the initial period after aortic occlusion when the collaterals were not yet fully developed and this first period of relative ischemia might even have precipitated a formation of some new vessels besides the structural adaptation of the preformed ones

Discussion

It is generally agreed that the hemodynamics of hypertension is characterized by an increased vascular reactivity and a raised basal resistance which has been widely assumed to directly reflect an increased level of smooth muscle activity For such reasons hemodynamic analyses have in most cases set out from this level of resting tone However to reveal the true hemodynamic situation it is as has been earlier re peatedly stressed (Folkow 1956 Folkow *et al* 1958 1970 a b Sivertsson 1970 Folkow 1971) necessary to start rather from the baseline set by the resistance at *complete* vascular relaxation The reason is that the profound hemodynamic in fluence that can be exerted by prevailing structural vascular changes will otherwise easily escape detection It should in this context be stressed that *e g* media hyper trophy of the resistance vessels is as likely to take place in hypertension as the general ly acknowledged hypertrophy of the left ventricle and has moreover been described for decades by histologists the most convincing study being perhaps that provided by Furuyama (1962)

Hemodynamic studies of essential hypertension in man (Folkow *et al* 1958 Conway 1963 Sivertsson 1970) and of spontaneous hypertension in rats (Folkow *et al* 1970 a b) have provided strong evidence of a structural adaptation of the resistance vessels This structural change seems to be marked enough to alone ex plain virtually the entire resistance increase in the resting equilibrium For example the spontaneously hypertensive rat (SHR) exhibits an increased resistance of the entire systemic circuit even when the vessels are completely relaxed (Folkow *et al* 1970 a) which is also the case in the forearm and hand of hypertensive man (see Sivertsson 1970) This increase is as large or almost as large as the pressure increase during resting conditions Moreover the resistance vessels of the SHR hindquarter preparation exhibit a series of characteristic hemodynamic changes which can in detail be explained by a media hypertrophy proportional to the pressure rise and encroaching on the lumen even at complete vascular relaxation In fact no other vascular change structural or functional can alone explain all these hemodynamic characteristics several such factors must then be assumed to be present simultaneous ly and the hemodynamic influence of the well documented media hypertrophy must be disregarded (Folkow *et al* 1970 b) These circumstances make it highly likely that the structural changes mentioned are almost solely responsible of the raised

resistance in well established primary hypertension at least in the "resting" equilibrium

The mentioned type of structural adaptation of the resistance vessels will raise the "baseline", from which vascular activity sets out and also create a characteristic vascular hyperreactivity which does not call for any changed smooth muscle characteristics apart from a mere increase in bulk of this wall compartment Such an adaptation must further be considered as an exponent of a normal tissue response to a change of load the most striking vascular example of this being perhaps the structural changes occurring in veins used as arterial grafts The trouble is however that such *per se* normal adaptations of vascular structure will have serious hemodynamic consequences once the entire arterial tree has become affected as a response to an increased load that may well be intermittent in type and functional in origin As a result the resistance equilibrium and the range of dilatation constriction becomes reset to a higher level Because of the consequent vascular hyperreactivity this may invite a vicious circle in case smooth muscle activity is not reduced below normal for some period which may allow for a regression of the changes

The time needed for such a structural adaptation to be completed is obviously of considerable interest *e g* for evaluations whether it is of importance for the establishment and maintenance of a hypertensive state or whether it is so very slow as to be merely a late complication of the hypertensive state In case it can occur within few weeks it is likely to be so intertwined with functional perhaps intermittent trigger mechanisms that it may be of key importance for the very creation of a truly hypertensive state Folkow and Sivertsson (1968) observed in cats that the arterial vessels within 3 weeks markedly adapted their design to an altered load as judged from both structural and hemodynamic changes In the present study a state of chronic hypotension was established at an early age and for varying periods in the hindquarters of both SHR and NCR in order to further explore the role of a regionally altered pressure level It was considered to be of particular interest to see whether this could completely annul the characteristic signs of media hypertrophy otherwise occurring in the SHR resistance vessels (Folkow *et al* 1970 b) Perfusion of the isolated hindquarters of SHR and NCR which had been in a state of hypotension for 6—16 weeks because of a ligation of the abdominal aorta with collateral bypass revealed the following main differences to ordinary SHR and NCR with respect to the resistance vessels

1 A decrease of resistance at maximal dilatation which if anything was *more* pronounced than the reduction in regional pressure as measured from the tail artery before the experiment (Fig 2) This indicates a marked structurally based widening of the resistance vessels perhaps also a formation of new vessels as a local response to the relative ischemia present just after the aortic ligation when collateral had not yet been fully developed

2 A decrease of the maximal contractile strength and of the steepness of the resistance curve associated with a displacement of the M_{50} to the right (Fig 1) These changes of the resistance vessels were largely proportional to the region

duction in pressure thus closely adapting the wall/lumen ratio to this lowered pressure level They cannot be ascribed merely to any new formation of otherwise normal vessels, largely the entire population of resistance vessels must have been affected Smooth muscle sensitivity on the other hand appeared to be largely the same in all groups of animals whether their hindquarter pressures had been normal lowered or raised

These clear signs of a structural vascular adaptation to a regionally reduced pressure were evidently well established already within few weeks and in the SHR hindquarters they completely prevented the hypertrophic changes of the resistance vessels that ordinarily take place in these animals (Folkow *et al* 1970 b) Since in each animal the hypotensive hindquarter vessels must have been exposed to largely the same neurohormonal influences as the other systemic vessels but nevertheless adapted their structure closely to the regional pressure level in both the aortaligated SHR and NCR it appears that the regional pressure load really is the prime determinant of the structurally based wall/lumen ratio of the resistance vessels

It was however mentioned above that the reduction of flow resistance at maximal dilatation appeared to be more extensive than the adaptation of the vascular walls in the hypotensive NCR SHR hindquarters For example when compared with ordinary NCR the first parameter was reduced by about 40 % but the latter one only by some 25 % (see Fig 2) If however ordinary SHR and NCR are similarily compared the same two parameters were 35 % and 40 % higher in SHR The quantitatively different relationship between them in the hypotensive hindquarters may have the following background Initially the aortic ligation must cause a marked reduction of the maximal blood flow capacity to the hindquarters and even imply a state of relative ischemia before collateral development has efficiently overbridged the obstruction The initially threatened nutrition may well cause not only a marked compensatory luminal growth of existing vessels but also trigger some formation of new vessels contributing to the markedly reduced resistance at maximal dilatation

The wall thickness of the individual resistance vessel on the other hand appears to adjust itself primarily to the current level of wall tension as expressed by Laplace's law As to the main wall element of such a vessel the smooth muscles their structural adaptation is probably also greatly dependent of their average activity level which in turn is mainly a consequence of their autoregulatory adjustments to the current pressure and metabolic situation For such reasons an average rise in pressure would as to its main structural consequence for the resistance vessels lead to a hypertrophic media adaptation This evidently in part encroaches upon the lumen even at maximal dilatation inevitably raises the wall/lumen ratio and markedly changes the hemodynamic characteristics towards a state of vascular hyperreactivity which may well be combined with a perfectly normal smooth muscle reactivity

To summarize the present results are probably but one example of how mesenchymal tissues in general readily and rapidly adapt their structure to changes in load However the results illustrate the most important hemodynamic consequences that such *per se* normal adaptations will have with respect to the precapillary

vessels where resistance to flow varies inversely to the fourth power of the internal radius and where vascular dynamics are markedly affected by the large bulk of contractile wall elements It further seems clear that such a structural adaptation can occur so rapidly that it is likely to be involved in the very initiation of primary hypertension and that it seems to constitute the main element for maintenance of this disturbance, being probably also involved in other types of hypertension Moreover, it implies a possibility of a vicious circle if not effectively counterbalanced, but may also offer good chances for a true regression, if only pressure is kept strictly at or below the normal level for a sufficient time period

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Pulmonary Blood Volume and "Pulmonary Hematocrit" during Hypervolemia in Rats

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Abstract

Aarseth, P. *Pulmonary blood volume and "pulmonary hematocrit" during hypervolemia in rats.* Acta physiol. scand. 1971. *83.* 542—553.

In rats erythrocytes and plasma albumin were labelled by radioisotopes and circulatory arrest was then achieved by rapidly freezing the intact animal in liquid nitrogen. Pre-mortal blood content in different organs could then be evaluated by measuring the radioactivity *in tissue* samples from the frozen animals. In one group of rats the circulating blood volume was increased 10 % by blood transfusions. The pulmonary blood volume in these hypervolemic animals was found to increase by 80 %. Most of this increase was due to an augmentation of the pulmonary plasma content, consequently "pulmonary hematocrit" had decreased markedly. It could be demonstrated that this increase in pulmonary blood volume was caused by the hypervolemia *per se*, and not by substances released from or by the transfused blood. When the left thoracic vago-sympathetic nerve trunk had been cut beforehand, no increase in lung blood volume was found in hypervolemic rats. It is suggested that the increase in lung blood volume normally found after a blood transfusion could stem from a constriction of small vessels. This could then lead to a passive distension of vascular sections upstreams to the constricted ones.

It has previously been demonstrated in the rat that subsequent to a blood loss there is a more marked reduction in the pulmonary blood volume than in the blood volumes of several other organs (Aarseth 1970). This marked lung blood volume reduction did partly depend on a normal pulmonary innervation (Aarseth 1971). These findings prompted the present investigation on how the pulmonary vasculature in rats participates in blood accomodation during hypervolemic situations. Using an indicator dilution technique DePasquale, Hyman and Burch (1965) and also Kerr and Kirklin (1970) have found a large increase in pulmonary blood volume in dogs where the circulaing blood volume had been rapidly increased. However, with the same type of technique de Freitas *et al.* (1965) and Korsgren (1966) could not unveil any increase in lung blood volume during induced hypervolemia in man. This discrepancy between findings in the dog and in man may be due to species differences. However, the method used is not a satisfactory one for volume evaluations in different circulatory situations.

In the present investigation pulmonary blood volume of hypervolemic rats has been evaluated with a different type of method Whole anesthetized rats were rapidly frozen in liquid nitrogen Erythrocytes and plasma albumin had been labelled beforehand by radioisotopes, and the pulmonary blood volume could then be estimated from the radioactivity in the excised frozen lungs Immediately after a blood transfusion an extra amount of fluid which equaled most of the transfused blood was found to be present in the lungs This degree of fluid accumulation in the lung depended on an intact sympathetic innervation to the organ

Methods

Male albino Wistar rats, weighing 210—240 g, were used They had free access to food and water until the experiments were carried out

blood sample and in the tissue samples (Aarseth 1970)

In one group of animals the injections of ^{51}Cr labelled erythrocytes was omitted These

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as usual, the labelled albumin was, however, not injected until 2 min before the animals were immersed in liquid nitrogen In these experiments the blood sample for measurement of the radioactivity in the circulating blood was not withdrawn until immediately before the freezing of the animal In some other animals the isotope-injection, blood transfusion and the blood sampling were performed as usual, but the freezing was omitted In these experiments the chest was opened and the lung hilus clamped as fast as possible The lungs were then removed and their content of radioactivity measured.

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The procedure for denervation of the lung has been described in detail elsewhere (Aarseth 1971) The left thoracic vago-sympathetic nerve trunk was cut behind the left auricle This necessitated a thoracotomy and the animals were allowed a 4 days recovery period before a blood volume experiment was carried out In another group of animals the left cervical vago-sympathetic nerve trunk was cut in the neck This cutting of the nerve was carried out just before the blood volume estimations were performed

Arterial bleeding was carried out in some animals during the transfusion period The ri femoral artery was cannulated and blood was withdrawn from it using a Harvard du

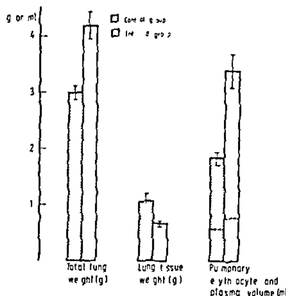


Fig 1 The effect of blood transfusion on total lung weight on calculated weight of lung tissue proper and on pulmonary blood volume The one group represents non transfused control animals (from data published elsewhere Aarseth 1970) the animals in the other group had received a transfusion of 1 4 ml whole rat blood 5 min before freezing All differences are significantly below 0 1 % level Bars on the top represent ±S E

In this and in the following 2 figures the columns representing pulmonary blood volume are divided by a stipled line The part below the line represents the erythrocyte volume and that above the line the plasma volume

drawal infusion pump (Model 947) Blood was withdrawn at exactly the same rate as that of the infusion into the right femoral vein

Hematocrit in blood from large vessels was obtained from the withdrawn blood sample using a microhematocrit centrifuge *Pulmonary hematocrit* was defined as the lung erythrocyte volume/(lung erythrocyte volume + lung plasma volume) *Wet lung weight* was obtained by subtracting the calculated weight of pulmonary blood from the total lung weight the specific weight of blood was taken to be 1 05

Microscopic examinations were performed on lungs from 1 control animal and from 2 hypervolemic animals They were treated exactly as the other animals as regards isotope injection transfusion blood sampling and freezing One of the lungs was removed from the frozen animal for blood volume estimation Sections were made from the other frozen lung by freeze-cutting and staining was carried out with eosin and hematoxylin

Wilcoxon's two sample test was used for evaluation of the significance of differences between groups

Results

The aim of the present investigation was to evaluate the degree to which the pulmonary vascular bed in the rats increases its volume during induced hypervolemia Hypervolemia was induced in 7 rats by transfusing each of them with 1 4 ml of blood This gave a blood volume increment of about 10 % When these animals were compared to a control group of non transfused animals the following observations were made (Fig 1)

1 The mean total weight of the lungs increased from 3 0 to 4 2 g
2 The mean pulmonary blood volume as estimated from the organ content of radioactive tracers increased from 1 85 to 3 38 ml
3 This large increase in lung blood content was almost exclusively due to an apparent pulmonary accumulation of plasma Actually the pulmonary hematocrit was reduced from 70 % to 49 % of that in blood from large vessels

TABLE I Lung weights, pulmonary blood volumes and 'pulmonary hematocrits' in 6 groups of rats One group is a normovolemic control group In 4 groups the animals were made hypervolemic by different means, and in the last group the animals have simultaneously been bled and transfused at the same rates (S E in the parentheses)

	Control	I v infusion of 1 4 ml			I a infusion of 1 4 ml blood	I v infusion Arterial bleeding
		Blood	Plasma	0 9 % NaCl		
n	17	7	4	3	3	4
Lung weight (g)	3 02 (±) 0 12	4 18 (±) 0 26	3 80 (±) 0 13	3 46 (±) 0 28	4 92 (±) 0 32	2 41 (±) 0 10
Pulmonary blood volume (ml)	1 85 (±) 0 10	3 38 (±) 0 29	3 02 (±) 0 20	2 63 (±) 0 29	3 68 (±) 0 39	1 67 (±) 0 09
Pulmonary hematocrit as % of large vessel hematocrit	70 (±) 3	49 (±) 4	50 (±) 5	56 (±) 6	39 (±) 9	74 (±) 3

4 *The weight of the lung tissue proper, that is total lung weight minus weight of* calculated pulmonary blood volume (Specific weight of blood = 1 05), decreased from 1 07 g to 0 67 g subsequent to a transfusion.

It is remarkable that a sudden increase in total blood volume of 1 4 ml was immediately followed by an increase in lung weight of 1 2 g

In order to test whether this was a result of hypervolemia *per se* or of some other effect of the infusion of homologous blood the following experiments were performed

1 The rate of blood infusion was varied, but with the infusions always being finished 5 min before freezing of the animals The same weight increase (and plasma volume increase) was found whether the transfusions lasted for 30 5 or 1 min
2 Rat plasma or a 0 9 % NaCl solution was used for transfusion instead of whole blood (Table I) Also when hypervolemia was induced by these means, there *were large increases in lung weight and lung blood volume, and larger* with plasma than with saline
3 In one group of rats heparinized whole blood was infused i v and at the same time blood was withdrawn from the femoral artery at exactly the same rate In these animals pulmonary weight and blood volume did not differ from the values found in the control group (Table I)
4 In 3 rats the blood infusion was performed through a catheter in the femoral artery In these animals the effects on the lung were at least as marked as in the animals where i v infusions had been carried out (Table I

The large increase in plasma content in the lungs might reflect either an increase in the intravascular and or in the extravascular fluid compartment If fluid containing albumin was accumulated extravascularly plasma molecules larger tha

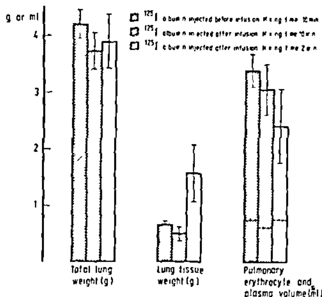


Fig 2 Total lung weight calculated weight of lung tissue proper and calculated pulmonary blood volume for 3 groups of rats All groups had received blood transfusions (the first and the third group 1 4 ml, the second group 1 ml only), but at different times Moreover, the ^{125}I albumin was injected at different times relative to the blood transfusions

The differences in total lung weights were not significant The third group differed significantly from the first one as regards tissue weight proper (p = 0 016) The difference in pulmonary blood volume between these groups was not significant (p ~ 11 %) Bars on top represent ± S E

albumin might possibly not reach this compartment In 6 rats ^{125}I-PVP (mean mol wt 160,000) was therefore used as plasma tracer together with ^{131}I human plasma albumin In all the rats the isotope mixture was injected 10 min before freezing In 3 of the rats a blood transfusion was performed as usual, lasting 5 min and being finished 5 min before freezing In the 3 other rats 1 4 ml of whole blood was infused in the course of 1 min and immediately before freezing In both these groups the weight of the lungs increased to the same extent and the pulmonary plasma content was large and almost the same whether calculated from ^{125}I-PVP or from ^{131}I albumin activity

In order to test whether this rapid pulmonary accumulation of labelled plasma molecules could take place only during the period where the fluid accumulation occurred the following experiments were performed In 4 rats labelled erythrocytes *were injected 10 min before freezing At the same time the* 5 min i v transfusion of whole blood was started Two min before freezing the dose of labelled albumin was injected and a blood sample of 0 2 ml was then withdrawn immediately before freezing In 3 other rats transfusions with 1 ml blood was performed as soon as the right femoral vein had been cannulated and 30—35 min before freezing Isotopes were injected 10 min before and a blood sample withdrawn 2 min before freezing The results found in these animals are shown in Fig 2, where they are compared with those from the group transfused in the ordinary way In all groups the lung weight increased to the same degree When the transfusion was performed firstly and the ^{125}I-labelled albumin then given and allowed a 10 min mixing period, then the pulmonary blood volume and the net lung tissue weight were comparable to the values found in the ordinarily transfused group When the ^{125}I-labelled albumin was given *after the transfusion but allowed only a 2 min* mixing period, then the

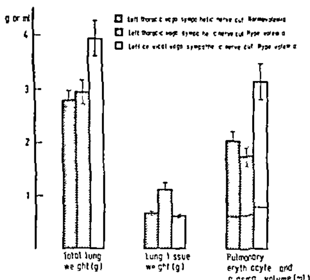


Fig 3 The effect of cutting the left vago-sympathetic nerve trunk upon lung weight and pulmonary blood volume subsequent to a blood transfusion The animals in the first and the second group have had their left thoracic vago-sympathetic nerve trunk cut The animals in the first group are normovolemic, while the animals in the second group had received a blood transfusion of 1 4 ml The difference in lung tissue weight between these groups was significant ($p < 0.001$) The animals in the third group had had their left cervical vago-sympathetic nerve trunk cut and had then received a 1 4 ml blood transfusion In this group the total lung weight and the pulmonary blood volume were significantly increased ($p = 0.024$ and 0.003 respectively), when compared to the hypervolemic group in which the thoracic vago-sympathetic nerve had been cut Bars on top represent ± S E

increase in the estimated pulmonary plasma volume was much smaller and the net tissue weight correspondingly larger

In another group of 3 animals isotope injection, i v infusion of whole blood and blood sampling were performed as usual, but instead of immersing the animals in liquid nitrogen their chests were opened The lung vessels were then clamped as quickly as possible, the lungs were removed and their content of radioisotopes estimated In these lungs there was found only a mean of 0 98 ml blood and the pulmonary hematocrit was 80 % of the large vessel hematocrit

It has previously been found that the pulmonary hematocrit in the rat is changed after a blood loss and that this change depends on an intact pulmonary innervation (Aarseth 1971) In the present experiments the pulmonary hematocrit was markedly reduced when the total blood volume of the rat was increased The mechanism responsible for this might also in some way depend on intact pulmonary innervation In order to test this possibility the left thoracic vago sympathetic nerve trunk was cut in 7 rats After a 4 days recovery period these animals were given a blood infusion of 1 4 ml and their pulmonary blood volume estimated in the usual way The results are shown in Fig 3 and compared to those from a control group of non transfused animals which had also had their left thoracic vago-sympathetic nerve trunk cut It can be seen that hypervolemia did not induce any increase in lung weight or in pulmonary blood volume when this nerve trunk had been cut On the contrary the plasma volume seemed to be reduced and the lung tissue weight correspondingly increased in th

In another group of 5 animals the left vago sympathetic nerve trunk was cut in the neck When transfusion was given to these animals, then the lung weight increased to a mean value of 3 96 g and the pulmonary blood volume to a mean value of 3 17 ml (Fig 3) These values were similar to those found in animals with intact pulmonary innervation

Histology When freeze cut sections of lungs from control animals and transfused animals were compared no apparent difference in the lung tissue could be seen

Discussion

The method used for estimation of pulmonary blood volume in the present series of experiments has the great advantage that it gives a direct measure of the blood content within the lungs Everett Simmons and Lasher (1956) demonstrated that there does not take place any redistribution of blood in the rat between the section below and the section above the diaphragm during freezing in liquid nitrogen This was so even if a couple of minutes elapsed before the temperature in the innermost organs in the rat was reduced to below 0° C The measured content of radioactive tracers within the frozen lungs can therefore be supposed to reflect the *in vivo* pre freezing situation

The infusion of 1 4 ml of whole blood to rats caused an increase in the lung weight of as much as 1 2 g an increment which equals about 40 % of the weight of lungs in control animals This increase was highly significant ($p < 0\,001$) (Fig 1) One could think of 3 possible explanations for his phenomenon Firstly insufficiency of the left heart with increased left atrial pressure could have developed due to the hypervolemia This is not very likely since the same increase in lung weight was found whether the transfusion with 1 4 ml blood was performed in the course of 1 5 or 30 min The slowest of these infusion rates corresponds to 0 2 ml/kg/min and is far below what is usually employed in clinical medicine

Secondly there could be some chemical effect of the infused blood *e g* release of vasoactive factors perhaps from blood platelets If however blood was removed from the animal at the same rate as new blood was i v transfused and the rat's blood volume thus kept unchanged then there was no effect on the lungs of the infused blood

A third and more likely possibility is that the effect found after transfusion was due to hypervolemia *per se* This possibility is supported by the findings of plasma and saline transfusions giving effects similar to those seen after blood transfusions The lesser effect of saline infusions may probably be explained by saline being a less effective volume expander than blood and plasma Furthermore the changes found in the lung were not depending on the transfusion being done through a vein The same increases of lung weight and of lung blood volume were found also when the infused blood was introduced through an artery so that it would firstly pass through systemic vascular beds

The weight increase of the lung in the hypervolemic rats (1 2 g) must reflect an increase in lung fluid content The ^{131}I albumin content in the lung increased to a similar degree and corresponding to about 1 4 ml of plasma It is most likely that both the fluid and the plasma albumin is found in the same compartment and really represent an augmentation of the plasma content of the lungs This extra plasma volume might theoretically be found intravascularly and/or extravascularly as edema The rapid accumulation of fluid in the lungs point to an intravascular localization The fluid augmentation within the lung could thus take place also when the whole transfusion was given in the last min before freezing Also when the lungs of hypervolemic rats were allowed to deflate before circulation through them was stopped then the pulmonary blood volume (and especially the plasma volume) was reduced to subnormal levels Since the lung vessels were here clamped within 30 sec of opening of the chest the large extra fluid content expected to be within these lungs must have been very rapidly transferred out of the organ Furthermore the 160 000 mol wt PVP was distributed in a volume in the lung which was just as large as that for radioactive albumin The lack of histological changes after transfusion also point to an intravascular fluid accumulation in hypervolemia

The decrease in calculated net lung weight in the hypervolemic rats is difficult to explain (Fig 1) Outward leakage of labelled plasma proteins would result in an apparent reduction in lung tissue weight It seems unlikely however that such a leakage has taken place to any great extent since PVP (mol wt 160 000) showed the same distribution as did albumin Further evidence against any marked extravascular deposition of radioactive albumin was found when the vessels in the excised lungs from one transfused animal were washed through with saline The remaining radioactive albumin in these lungs corresponded to only 0 1 ml of plasma — Another possible but somewhat strange explanation to the reduced lung tissue weight is that some inward flux of fluid has taken place after a transfusion

The pulmonary hematocrit found in rats which have been frozen in liquid nitrogen is rather low In normovolemic control rats it was found to be only 70 % of the large vessel hematocrit Rapaport *et al* (1956) used the Bradley equilibration technique in an attempt to estimate pulmonary hematocrit in the dog They found a value corresponding to 85 % of large vessel hematocrit Larsen (1966) used the same method in man and found the pulmonary hematocrit to be 94 % of the large vessel hematocrit The Bradley equilibration method is however not suitable for estimation of volumes in vascular beds with a high flow to volume ratio (Yu 1969) The Hamilton Stewart method has also been used for estimation of pulmonary hematocrit (Rapaport *et al* 1956 Parrish Strandness and Bell 1961) When this method was used very high values for pulmonary hematocrit was found corresponding to 95 % of the peripheral value In open chest cats pulmonary hematocrit has recently been found to be 68 % of the large vessel hematocrit when the lung vessels were clamped before estimation of their plasma and erythrocyte content (Aarseth and B 1971) The freezing of the rats has therefore not necessarily a falsly low p [illegible]

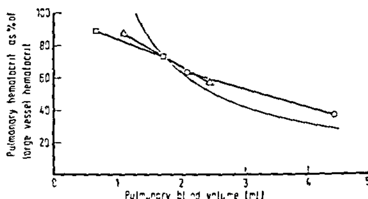


Fig 4 Pulmonary hematocrit as % of large vessel hematocrit in relation to pulmonary blood volume Data from 3 differently treated groups of rats

Control group (Aarseth 1970)
Δ $y = -22x + 111$ $r = 0\ 77 \pm 0\ 25$ (S.D)

Animals from which 12 % of the total blood volume had been withdrawn (Aarseth 1970
□——□ $y = -15x + 98$ $r = 0\ 68 \pm 0\ 21$ (S D)

Animals in which total blood volume had been increased by about 10 %
○——○ $y = -11x + 86$ $r = 0\ 81 \pm 0\ 21$ (S D)

The symbols on each line mark the extreme values of pulmonary blood volume in the respective group The curved line is the one to be expected if pulmonary erythrocyte volume was kept constant and changes in pulmonary hematocrit brought about by changes in plasma volume only

the advantage over other methods that it allows total mixing of isotopes and accurate measurement of isotope content in the lung to be carried out It is thus very likely that the present method gives a more correct pulmonary hematocrit value than the other ones mentioned above

In the hypervolemic rats in the present experiments a still lower "pulmonary hematocrit" was found It amounted to only 49 % of large vessel hematocrit In other experiments it has been found that the "pulmonary hematocrit" increases after a moderate bleeding (Aarseth 1970) The "pulmonary hematocrit" thus seems to be inversely related to the lung blood volumes

In Fig 4 is shown the regression lines for "pulmonary hematocrit" against pulmonary blood volume in 3 groups of animals In all groups (one normovolemic one hypovolemic and one hypervolemic) there is very good correlation between "pulmonary hematocrit" and pulmonary blood volume Furthermore the regression lines for the 3 groups are nearly identical This strongly suggest a common hemodynamic denominator for "pulmonary hematocrit" in variously treated rats such as e g the degree of axial streaming Axial streaming of erythrocytes is usually assumed to explain why "tissue hematocrit" (dynamic hematocrit) is lower than that found in large vessels Fåhraeus and Lindqvist 1931 measured dynamic hematocrit in glass capillaries 50 μ in diameter and found it reduced by 30 % when compared to that in the reservoir This reduction was accompanied by a reduced viscosity of the

blood Dintenfass (1967) has measured blood viscosity in still smaller capillaries and he found declining values down to a tube radius of about 5 μ The viscosity increased rapidly with further reduction in radius

The mean estimated pulmonary plasma volume increased by 1 325 ml and the mean pulmonary erythrocyte volume by 0 2 ml in the present series of experiments The 'hematocrit of the extra blood accomodated in the lung would then be only some 30 % of the large vessel hematocrit In small vessels the marginal layer of plasma is thought to be 1—5 μ thick (Bayliss 1962) It is thus possible to conceive a dynamic hematocrit as low as that found for the additional pulmonary blood in the present transfusion experiments in vessels with a diameter of 15—25 μ Recruitment of such small vessels could therefore explain the accumulation of low hematocrit blood in the lungs Also alteration of the diameter of a large number of small vessels could result in more marked axial streaming Another possibility could be that the thickness of the marginal plasma layer was generally increased as the flow rate increased and the vascular geometry became altered ¹³¹I albumin was ineffectively mixed with the post transfusion pulmonary plasma compartment when the tracer was injected only 2 min before the animals were frozen (Fig 2) This is not inconsistent with there being a large marginal layer of plasma in lung vessels in this situation One would not expect full tracer exchange against such a marginal layer to take place in a few passages of blood through the lung In this connection it should be mentioned that Rapaport *et al* (1956) found a much smaller plasma volume in the dog lung with the Hamilton Steward dilution method than with the Bradley equilibration method

The increment in total lung weight and pulmonary blood volume could be completely prevented by cutting the left thoracic vago sympathetic trunk (Fig 3) In this procedure both the sympathetic and the vagal fibres to the lung emerging from the left side are destroyed When however the left vago sympathetic trunk was cut at a cervical level leaving most of the sympathetic nerve supply to the lung intact (Daly and Hebb 1966) then transfusion had the same effect on the lung as in animals with intact innervation (Fig 1 and 3 The effect of denervation must therefore be connected with left sided sympathetic nerve fibres There are several ways in which alteration in activity in such nerves could result in an increased lung blood volume Firstly a reflex reduction in vascular tone could have such an effect Sympathetic fibres are apparently responsible for some vascular tone in rat lung vessels since the pulmonary blood volume in normovolemic rats increased when the fibres were destroyed (Aarseth 1971) However a reflex reduction in vascular tone can hardly explain the large increase in lung blood volume seen in the present experiments since this volume increase was abolished after left sided denervation A second possibility could be that lung vessels are reflexly and actively dilated upon a transfusion The cutting of vaso dilating nerve fibres would interfere with lung blood volume in the way observed In the dog Daly *et al* (1948) have demonstrated the presence of sympathetic vaso dilating nerve fibres to the lung They fibres in the left sided outflow only and not on the right side It is

prising that in the present experiments there was such a striking effect on total pulmonary blood volume of a one sided denervation There is no ready explanation for this phenomenon but the left side sympathetic innervation to the lung could here possibly dominate over the right sided one Cutting of the right vago-sympathetic trunk was not attempted in the present investigation because of the anticipated marked effect on the heart of such a procedure

A third possible explanation of the nerve dependent post transfusion increase in lung blood volume could be that increased vaso-constriction occurred in certain vascular segments This would lead to passive distension of vessels upstreams to the constricted ones Such a situation would be in accordance with the findings of De Pasquale *et al* (1965) These investigators found a larger pressure increase in small pulmonary veins than in the larger ones after induced hypervolemia in dogs At the same time they observed a large increase in pulmonary blood volume and a reduced total vascular resistance

Altogether the present experiments show that the lungs in the rat accomodate large extra amounts of low hematocrit blood just after a blood transfusion The nature of the vascular alterations involved cannot be definitely defined but nervously controlled vasomotor events and an increased degree of axial streaming appears to take place

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Differences in the Antidiuretic Response to Intracarotid Infusions of Various Hypertonic Solutions in the Conscious Goat

By

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Abstract

Eriksson L O Fernandez and K Olsson *Differences in the antidiuretic response to intracarotid infusions of various hypertonic solutions in the conscious goat* Acta physiol scand 1971 *83* 554—562

Various hypertonic equi-osmolal solutions were infused via permanently implanted catheters into the external carotid artery of conscious hydrated goats The infusions (1 5 ml/min) of 1 M NaCl 2 M fructose and 2 M sucrose caused a marked inhibition of the water diuresis The effect was evidently due to a release of antidiuretic hormone (ADH) since it disappeared after induction of diabetes insipidus No antidiuretic response occurred during infusions of either 2 M glycerol or 2 M galactose while a weak response was seen after infusions of 2 M glucose Infusions of 2 M urea gave inconsistent results Infusions of hypertonic fructose into the 3rd ventricle of the hydrated goat did not change the course of the water diuresis whereas the corresponding infusion of equi osmolal NaCl effectively released ADH It is concluded that neither changes in the blood osmolalities as such nor different penetration rates across the blood brain barrier can explain the results some of which appear incompatible with the current osmoreceptor theory

The antidiuretic response to intracarotid infusions of hypertonic sodium salt and sucrose solutions in the hydrated dog suggests that hypothalamic osmoreceptors regulate the release of antidiuretic hormone (ADH) from the neurohypophysis (Verney 1947 Jewell and Verney 1957) However hypertonic solutions of sucrose (Olsson 1969) and glucose (Andersson Olsson and Warner 1967) have no antidiuretic effect when infused into the 3rd brain ventricle of the goat whereas similar infusions of hypertonic NaCl effectively release ADH (Andersson, Dallman and Olsson 1969) These findings in the goat appear incompatible with the idea that osmoreceptors located *inside* the blood brain barrier regulate the release of ADH It was of interest therefore to study the effect of these and other osmotic stimuli when applied outside the blood brain barrier in the hydrated goat

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Methods

Animals 8 adult female goats (b wt 28—36 kg) were used The animals were routinely con

ted in these cages The

received 300 g of com-

expts over periods from

was 3 days

(0 8 mm i d , 1 2 mm o d) was implanted into the external carotid artery via its facial branch as was previously done in the sheep However, in the goat, it was very difficult to proceed via the facial artery Instead, the polyvinyl catheters were implanted via the superficial temporal branch of the carotid during all following operations The extravascular part of the catheter was covered by and glued to a close-fitting polyvinyl tubing, which was anchored with a suture close to the catheter's entrance into the artery Under X ray control, the tip of the catheter was placed in the external carotid artery about 2 cm caudal to the entrance of the internal maxillary artery To make the catheter X-ray opaque, and to ascertain that the subsequent infusions would reach the internal maxillary artery, a contrast medium (Urografin 60 %, Schering) was injected The catheter and its covering tube were then brought under the skin to the base of one horn and were taped onto it Using the same technique, a polyvinyl catheter for control infusions was implanted into the jugular vein, via its superficial temporal branch in one of the animals All implantations were made under general anesthesia (Nozinan "Leo" followed by nembutal) The catheters were flushed daily with isotonic saline, after which they were filled with heparine solution (5000 IE/ml 'Vitrum)

Implantations into the 3rd ventricle In two goats a permanent stainless steel cannula was implanted into the dorso-anterior part of the 3rd cerebral ventricle under general anesthesia as described previously (Andersson *et al* 1967)

Infusion technique A polyethylene tube connected to a syringe and fitted on to a perfusion apparatus ("Unita/Perfusor) was filled with the solution to be infused The polyethylene tube taped to one horn of the animal was flexibly suspended by use of a spring balanced wheel, and was connected to the vascular catheter by a stainless steel cannula In this manner the animals retained their original freedom of movement during the infusions

During infusions into the 3rd ventricle an inner cannula filled with the infusion solution and precisely fitting the permanent cannula, was inserted to the bottom of the permanent cannula The inner cannula was connected to a syringe with a polyethylene tube as described above

plete interruption of the neural connection between the hypothalamus and the neurohypophysis was obtained by passing radio-frequency current between the uninsulated electrode tips heating the tissue to about 65° C

Hydration The water diuresis was established by giving the goats by stomach tube about 100 ml of 38° C water/kg b wt into the rumen

Blood and urine samples Blood samples were obtained in heparinized syringes via a polyethylene tube inserted into the jugular vein on the infusion side Using this tube blood could be withdrawn while the animal was under a minimum of restaint Blood samples were routinely taken as follows 1) before hydration 2 at the onset of water diuresis 3 immediately before start of the infusion 4) 25 min after the onset of the infusion and 5 about 30 min after cessation of the infusion The urine was collected in 10 min samples via a retention catheter inserted in the urinary bladder

Analyses Plasma and urine osmolalities were determinted by measuring the freezing depression using an 'Advanced Instruments INC osmometer or a Knauer
Since the mean preinfusion plasma osmolality was 290 mosm kg (Table I) this va
sistently used for the calculation of renal free water clearance

TABLE I Effects of the given water load (100 ml kg b wt) on the plasma osmolalities of 6 goats used for intracarotid infusions

Sample	Number of samples	Mean and (SD) mosm/kg
Pre hydration	16	294 (5)
At the onset of water diuresis	16	287 (5)
Immediately before infusion	22	290 (5)
Post infusion	21	291 (5)

Results

Intracarotid infusions in the normal hydrated goat

Preliminary intracarotid infusions revealed that hypertonic NaCl had the most consistent and strongest antidiuretic effect of the osmotic stimuli applied in this study For this reason control infusions of hypertonic NaCl were made, following negative infusions of the non electrolyte solutions to ascertain whether the infusions had reached the appropriate part of the brain Such control infusions appeared necessary because a catheter permanently implanted into the carotid artery may reduce the blood flow through the vessel and thereby disturb the normal ipsilateral blood supply to each half of the brain (Baldwin and Bell 1963) In this study two of the goats did not respond with antidiuresis as long as they had only one carotid artery *provided with a catheter Hence another catheter was implanted into the contralateral* carotid artery in these animals The expected antidiuretic response to infu*sions of hypertonic NaCl was then obtained in both goats regardless of whether the* infusion was made into the left or the right carotid artery

Preliminary expts also confirmed the observation made in sheep (Olsson and McDonald 1970) that at this water load (100 ml/kg b wt) the intracarotid infusion of hypertonic NaCl does not induce antidiuresis during the early stage of water diuresis Therefore all the infusions were made when the goats had excreted about a third of the water load At this stage the water diuresis had reached plateau level and the plasma osmolality had attained temporary equilibrium after a *minimum* value at the onset of the water diuresis (Table I)

With one exception (glycerol) the intravascular infusions were made at a rate of 1 5 ml/min The infusion periods were generally 30 min Only sucrose was infused *for shorter periods (see below*

Sodium chloride All 6 catheter goats were repeatedly subjected to intracarotid infusions of hypertonic (1 M NaCl The infusion of NaCl always induced a marked antidiuretic response with a negative renal free water clearance lasting for at least 40 min (Fig 1) During these infusions plasma osmolality rose an average 10 mosm/kg above pre hydration level in the blood taken from the ipsilateral jugular vein (Fig 2)

Fructose Three of the goats were subjected to infusions of 2 M fructose (4 expts) As shown in Fig 1 these infusions had an antidiuretic effect similar to that obtained during the corresponding infusions of 1 M NaCl Ipsilateral jugular plasma

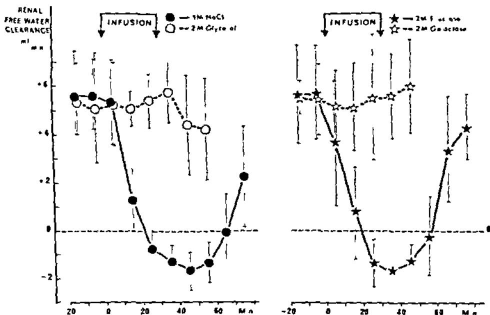


Fig 1 *Differences in the antidiuretic response to intracarotid infusions of various hypertonic equi osmolal solutions in hydrated goats* The infusion of 1 M NaCl and 2 M fructose (30 min, 1 5 ml/min) (*dark symbols*) have a marked antidiuretic effect (renal free water clearance turned into negativity for about 40 min) The corresponding infusion of 2 M glycerol and 2 M galactose (*open symbols*) do not release ADH (unchanged renal free water clearance) Each symbol represents mean and the vertical bars standard deviations of the measurements No expts NaCl = 13 (6 goats), glycerol = 4 (2 goats) fructose = 4 (3 goats) galactose = 5 (3 goats)

osmolality rose an average 7 mosm/kg above pre-hydration level during the fructose infusions (Fig 2)

Galactose 3 of the goats were used for intracarotid infusions of 2 M d galactose (5 expts) The infusions did not change the course of the water diuresis Hence, renal free water clearance remained constant during and for at least 20 min after the infusions (Fig 1) A mean increase in the ipsilateral jugular plasma osmolality to 11 mosm/kg above pre hydration level was obtained during the infusions of hypertonic galactose (Fig 2)

Glucose Two infusions of 2 M d glucose were made in one of the goats Only a slight decrease in the renal free water clearance was observed (from +6 to +3 ml/min) Ipsilateral venous plasma osmolality rose 11 mosm/kg above the pre-hydration level during the intracarotid infusion of hypertonic glucose

Glycerol Infusions of 2 M glycerol at the usual rate were made in two of the goats (4 expts) The course of the water diuresis did not change in any of these expts (Fig 1) However the rise in ipsilateral jugular plasma osmolality obtained during the infusions of glycerol was less than that seen during corresponding infusions of other hypertonic solutions An osmolality only slightly above pre-hydration level

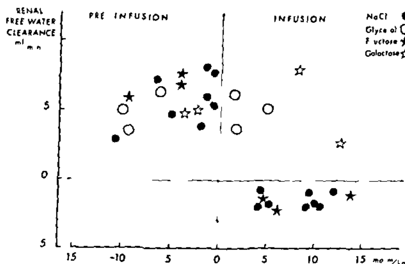


Fig 2 Effects on renal free water clearance and on ipsilateral jugular plasma osmolality of intracarotid infusions of various hypertonic equi-osmolal solutions in the hydrated goat Note that an equivalent rise in blood tonicity obtained by chemically related substances (monosaccharides) is either ineffective (galactose) or effective (fructose) as stimulus for ADH release (= negative renal free water clearance) Renal free water clearance values represent the mean of two 10 min periods immediately prior to the infusions (Pre infusion) and the mean of three periods 20—50 min after the start of the infusion (Infusion) The plasma osmolality (abscissa) is expressed as deviation from the pre hydration level = 0 The ipsilateral jugular blood samples were taken 15 min before the infusion (Pre infusion) and 5 min before the end of the 30 min infusion period "Infusion) Infusion rate = 1 5 ml/min with the exception of one glycerol infusion 2 ml/min) Non electrolyte solutions 2 M NaCl solution 1 M

reached possibly due to the rapid turn-over of glycerol in the blood (Hagen 1963) *Therefore the rate of infusion of 2 M glycerol was increased to 2 ml/min during a* 30 min period in an additional expt Although this infusion incre[illegible] silateral venous plasma osmolality above the pre hydration level by 6 mosm/[illegible] diuretic response was obtained However a subsequent infusion of 1 M N[illegible] caused less increase in plasma osmolality had appreciable antidiuretic ef[illegible]

Urea Intracarotid infusions of 2 M urea gave inconsistent resul[illegible] three animals used the course of the wate[illegible] is did not cha[illegible] goat responded *with marked antidiuresis* [illegible] diate effect the third animal The same marked rise [illegible] venous pl[illegible] (13 mosm/kg above pre hydration level) w[illegible] first tw[illegible] tioned

Sucrose During all the expts mentioned above [illegible] not seem to take notice of the infusions Howev[illegible] were infused into the carotid artery the anima[illegible] started *to pant* Therefore the infusions were int[illegible] ther sucrose infusions were made in this or other g[illegible] by marked antidiuresis

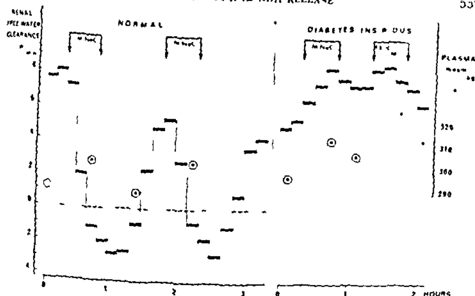


Fig 3 *Left* Marked inhibition of the water diuresis elicited by the intracarotid infusion of 1 M NaCl in a hydrated goat when its hypothalamo neurohypophyseal system was still intact
Right Complete lack of antidiuretic response to intracarotid infusions of 1 M NaCl and 2 M fructose when diabetes insipidus had been induced in this goat by interruption of the neural connection between the hypothalamus and the neurohypophysis Since the animal is not hydrated the plasma osmolality in the ipsilateral jugular vein blood rose to a considerably higher level during these infusions than during the infusions performed in the intact hydrated animal
Rate of infusion 1 5 ml/min

Intravenous infusions

Intrajugular control infusions were made in one of the goats to ascertain whether the strong antidiuretic effect obtained during the intracarotid infusions of 1 M NaCl and 2 M fructose was centrally elicited In contrast to the intracarotid infusions the corresponding infusions into the jugular vein caused no or only a weak and much delayed inhibition of the water diuresis

Intracarotid infusions in the diabetes insipidus goat

Expts performed in the animal having thermocouple electrodes implanted bilaterally in the median eminence region produced direct evidence that the inhibition of the water diuresis obtained by the intracarotid infusions in fact was due to a release of ADH from the neurohypophysis

Before lesioning the expected pronounced inhibition of water diuresis was obtained by intracarotid infusions of 1 M NaCl in the hydrated animal (Fig 3 *left*) The neural connection between the hypothalamus and the neurohypophysis was then interrupted by radio frequency heating of the median eminence When the transitory stage of diabetes insipidus was fully developed with a conspicuous water diuresis in the non hydrated animal intracarotid infusions of 1 M NaCl and 2 M

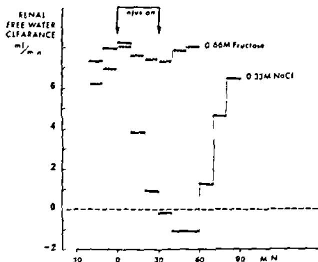


Fig 4 Lack of antidiuretic response to an infusion of hypertonic (0 66 M) fructose (10 μl/min, 30 min) into the 3rd brain ventricle of a hydrated goat. For comparison is shown the strong antidiuretic effect obtained in the same animal in response to the corresponding infusion of equi-osmolal NaCl solution

fructose no longer had any antidiuretic effect Rather, some increase in renal free water clearance occurred during the infusion periods (Fig 3, *right*) Since the goat was not hydrated during the transient stage of diabetes insipidus, the infusions of NaCl and fructose induced thirst, and caused a considerably greater increase in plasma osmolality than that seen during corresponding infusions in the intact hydrated animal (Fig 3)

Infusions into the 3rd brain ventricle

Since intracarotid infusions of hypertonic fructose effectively elicited ADH release in the hydrated goat it was of interest to study whether the same stimulus would have this effect when also applied inside the blood-brain barrier Hypertonic (0 66 M) fructose was infused into the 3rd ventricle of two hydrated goats at a rate of 10 μl/min for 30 min The infusions did not change the course of the water diuresis However corresponding infusions of equi-osmolal (0 33 M) NaCl had a strong antidiuretic effect in these animals (Fig 4)

Discussion

The current concept of an osmometric regulation of the release of ADH from the neurohypophysis originates from Verney s fundamental studies (Verney 1947, Jewell and Verney 1957) Verney found that a rise in the tonicity of the blood which flows through the anterior hypothalamus and adjacent parts of the brain may cause a release of ADH in the hydrated dog This happened when hypertonic solutions of sodium salts or of sucrose were used to increase the blood tonicity However, when hypertonic urea was employed for the same purpose no release of ADH was obtained On the basis of these experiments, Verney suggested that "osmoreceptors"

in the hypothalamus regulate the release of ADH He emphasized that these receptors are probably not stimulated by a rise in blood tonicity as such but rather by changes in the extracellular fluid which by dehydration reduce the volume of the receptors This would explain why urea which rapidly diffuses into the cells was not found to stimulate the osmoreceptors

Verney did not raise the question as to whether the osmoreceptors are located inside or outside the blood brain barrier and did not discuss what influence brain barrier systems may have on the effect of blood borne osmotic stimuli Both problems are actualized by this study in the goat which especially stresses two features of the ADH releasing mechanism 1) Hypertonic solutions of certain saccharides (fructose and sucrose) act as very effective stimuli for ADH release when infused into the carotid artery, but have no such effect when applied inside the blood brain barrier (in the 3rd ventricle) 2) An equivalent rise in blood tonicity obtained by various chemically related substances (like monosaccharides) may be either effective or ineffective as stimulus for ADH release (Fig 2)

At a cursory glance these observations seem to indicate that the rate of penetration over the blood brain barrier may determine how effectively various blood borne osmotic stimuli cause a release of ADH A rapid rise in carotid blood tonicity, induced by a substance which penetrates the blood brain barrier very slowly is likely to cause temporary dehydration of the brain This would act as a stimulus to osmoreceptors in Verney s sense provided that the receptors are located inside the blood brain barrier Information appears to be lacking about the characteristics of the blood brain barrier of the goat However to judge from studies in other mammalian species the difference in the antidiuretic response obtained during intracarotid infusions in this study cannot be explained exclusively by dissimilarities in the rate at which the various substances penetrate the blood brain barrier Studies in other species have revealed that the blood brain barrier is practically impermeable to fructose (Crone 1965 a) and sucrose (Crone 1963 Yudilevich and De Rose 1971) It is true that both substances acted as effective stimuli for ADH release when infused in hypertonic solutions into the carotid artery of the goat However an almost as effective blood brain barrier appears to exist for glycerol and urea (Crone 1965 a Yudilevich and De Rose 1971 Kleeman Davson and Levin 1962) Yet no ADH release was obtained to infusions of hypertonic glycerol and the response to infusions of urea was inconsistent Glucose and probably galactose are transported from the blood to the brain by a rate limiting carrier mediated transport mechanism (Crone 1965 b Csaky and Rigot 1968) It must be expected therefore that the intracarotid infusions of these monosaccharides in hypertonic solutions caused a transport of water from brain to blood Nevertheless the intracarotid infusions of glucose had very weak antidiuretic effect and no inhibition of the water diuresis was obtained during the infusions of hypertonic galactose (Fig 1

The possibility has to be considered that osmoreceptors in Verney s sense are located outside the blood brain barrier or in a region of the central nervous system which lacks an effective blood brain barrier If so the results of this study

indicate that the membranes of the osmoreceptors are considerably less permeable to fructose than to the other monosaccharides used for intracarotid infusions This would also mean that the ADH release which is obtained by the infusion of minute amounts of hypertonic NaCl into the 3rd ventricle (Andersson *et al* 1969) is not due to osmoreceptor stimulation, since the corresponding infusions of equi-osmolal solutions of sucrose (Olsson 1969) and of fructose (Fig 4) do not release ADH

Recent studies of a central sodium angiotensin interaction in the goat indicate that Na^+ sensitive receptors, near the 3rd ventricle, participate in the regulation of thirst and ADH release It has been suggested that the activity of these receptors is predominantly influenced by the Na^+ concentration of the cerebrospinal fluid (*cf* Andersson 1971) At present it cannot be excluded that a receptor system of this kind might have been stimulated by the ADH releasing intracarotid infusions These infusions may have affected the formation of the cerebrospinal fluid in such a manner that its Na concentration increased

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Perfusate Qualities and Spontaneous Edema Formation in an Isolated Perfused Lung Preparation

By

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Abstract

NICOLAYSEN, G *Perfusate qualities and spontaneous edema formation in an isolated perfused lung preparation* Acta physiol scand 1971 83 563—570

[illegible] h perfusion period when [illegible] Krebs Ringer solution was used as a perfusate at 38° C massive edema was always formed within 2 h of perfusion This development was somewhat retarded when the same perfusate was used at 28° C In confirmation of previous findings the presence of erythrocytes in the perfusate was found to prolong the survival time of the preparations The time interval prior to edema formation was thus significantly increased when erythrocytes were added to the perfusate of 4 % albumin in Krebs Ringer solution The effect was more marked with an hematocrit of 30—40 % than with one of 10—11 % The results indicate that the maintenance of normal capillary permeability is dependent upon some form of biochemical interplay between the perfusate and the capillary endothelium

It has long been recognized that edema tends to develop in isolated perfused lungs or heart lung preparations after variable time of perfusion (Newton 1932 Daly *et al* 1946) There are several reasons why such edema formation in isolated lungs might be subject to further and more detailed analysis Work in the field of pulmonary transplantation requires practical knowledge about good ex vivo preservation of lungs including data on the adequacies of perfusates used for such preservation (Veith *et al* 1968) — Also from a theoretical point of view one would like to know how and why edema develops or is prevented from being developed in isolated perfused organs like the lung

In an examination of this problem in isolated ventilated perfused rabbit lungs Lunde (1967) found the presence of erythrocytes in the perfusate to be [illegible] siderable importance for the maintenance of transvascular fluid bal[illegible]

With whole blood as perfusate edema did not develop in the lungs until after 7—9 h of perfusion However, when the lungs were perfused with plasma, they developed massive edema in the course of 2 1/2 h Some preliminary experiments performed by the present author on the same lung preparation as used by Lunde (1967) showed much more variable results with plasma as perfusate This prompted the present investigations on perfusate characteristics and development of edema in isolated, ventilated and perfused rabbit lungs Lungs are suitable organs for examination of edema formation and edema prevention Firstly the organ has a large capillary surface area and secondly edema formation in many instances is easy to detect as the amount of extravascular fluid accumulating can be large relative to the weight of the tissue

Methods

Rabbits of either sex, weighing 2—3 5 kg were anesthetized by i.v. injections of 30—50 mg/kg of pentobarbitone (Nembutal® Heparin 750 I U /kg (pure powdered heparin (Novo) dissolved in saline) was also given i.v. Through a tracheostomy positive pressure ventilation was started The thorax was then opened by a midline incision The caval veins were dissected free and ligated, and at the same time the ventilation was stopped The preparation consisting of the lungs the heart and a minimum of other mediastinal tissues was then removed Glass cannulas were placed in the pulmonary artery (through an incision in the right heart ventricle) and in the left auricle A string was tightened around the ventricles near the atrioventricular groove

Perfusion Some 9—14 min after the rabbit's own circulation had been stopped perfusion of the lung preparation was started A Dale and Schuster pump was used for the constant volume pulsatile (88 strokes per min inflow perfusion The pulmonary arterial pressure was continuously recorded by connecting a side branch of the pulmonary arterial tubing to a pressure transducer (Sanborn P23Db The perfusate appearing in the left auricle was drained back to the reservoir The left atrial pressure LAp) was kept constant throughout each perfusion and at 1—2 5 cm of water The first 25—50 ml of fluid leaving the outflow cannula was discarded in order to avoid contamination of the perfusate with stagnant blood from the perfused vasculature The flow through the preparation was measured by collecting the outflowing perfusate for 6 or 10 sec The flow was kept constant throughout every experiment and the flow level chosen for individual preparations varied between 200 and 400 ml/min

Ventilation Immediately following the start of perfusion the lungs were inflated and positive pressure ventilation started with a Starling "Ideal pump (34 strokes per min) The peak inspiratory pressure was 9—10 and end expiratory pressure 1—2 cm of water respectively A 5 % CO_2 in air mixture was used for ventilation In some instances the CO_2 percentage was decreased in order to keep the pH of the perfusate above 7.2

Weighing technique Via the string around the ventricles the preparation was suspended underneath a force transducer Sanborn FTA 100-1) The other side of the transducer axis was connected to a counter balance system Weight changes of the preparation down to 0.05 g could be recorded Calibration of the system was performed at the end of each experiment by placing suitable loads on the lungs A more detailed description of the preparation the weighing technique and the perfusion system has been given elsewhere (Nicolaysen 1971)

Edema detection Increase in preparation weight was used as indication of edema formation Edema development and the accompanying weight increase followed an accelerating course a fact which helped in discerning intravascular volume changes and the extravascular accumulation of fluid in edema The weight of the lung tissue proper in these preparations is about 7 g and the intravascular volume in the inflated lungs is about 11 g (Lunde 1967) When the weight of the preparation had increased by 4 g from the lowest weight observed during the perfusion edema was said to be present (Lunde 1967) At this level of weight increase about 25 % weight increment moderate but manifest edema could be observed macroscopically on cutting through the lung tissue

Perfusates a) *Heparinized horse plasma* was obtained by centrifugation of heparinized 30 I U (ml) horse whole blood at about 1000 × g for about 10 min The plasma was divided in appropriate batches and frozen In some experiments the erythrocytes obtained by this procedure were also used (see below) About 1 h before start of an experiment a plasma portion was thawed at 38° C and filtered once through one layer of filter paper b) *A modified*

albumin Krebs Ringer solution (4 % AKR) containing per l (in mmol) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.5, KH_2PO_4 1.2, $NaHCO_3$ 24.8 The solution also contained 40 g of albumin (bovine Sigma) and 0.15 g of glucose per l and 30 000 I U of heparin (pure powdered heparin (Novo)) *c) 4 % AKR containing resuspended rabbit or horse erythrocytes* Rabbit erythrocytes were obtained from blood taken by heart puncture in anesthetized (30 mg/kg ... ...ood obtained was ...out 1000 × g and ...% AKR Horse ...d resuspended in 4 % AKR The hematocrits of these solutions were adjusted to the desired levels and controlled by measurements in an International microcapillary centrifuge, model MB

The perfusates were kept thermostated at 38±0.5° C or at 28±0.5° C during the perfusions The pH of the perfusate was measured intermittently during the perfusion and kept within 7.2—7.6 by adjusting the CO_2 percentage in the gas mixture used for ventilation

A certain spontaneous vasoconstriction developed in some experiments In these experiments m-cresol (10 μg/ml), adrenaline (0.25 μg/ml) or papaverine (20—70 μg/ml) was added, and the spontaneous vasoconstriction thereby effectively counteracted (Hauge, Lunde and Waaler 1966)

Statistical analysis The Wilcoxon two sample test was applied to evaluate the significance of differences between groups of experiments

Results

A) *Experiments at 38° C with horse plasma as perfusate* In 7 consecutive lung perfusion experiments horse plasma thermostated at 38° C was used as perfusate All batches of plasma were obtained from one and the same donor The pulmonary atrial pressure (PAp) did not exceed 24 cm of water in any experiment The development in weight and in PAp in one of these experiments are shown in Fig 1 The times from start of perfusion to the point where the preparations had gained 4 g in weight and thus reached a state of definite edema are listed in Table I It will be seen that the fate of these preparations varied considerably Four of them developed edema in the course of 1 1/2—4 1/2 h In the remaining 3 preparations a 4 g weight increase was not seen within the 6 h observation period (Table I) However in 2 of these experiments the weight had been increasing with time during the final 2 h of perfusion

B) *Experiments at 28° C with horse plasma as perfusate* 6 consecutive perfusions were carried out with horse plasma which was now thermostated at 28° C Again all plasma batches were obtained from one and the same donor horse All perfusions were followed for 6 h and the PAp did not exceed 20 cm of water in any experiment Papaverine had to be added in three of the experiments to counteract an initial spontaneous vasoconstriction None of these preparations developed edema during the observation period In the last h of perfusion the weight of all the preparations was either stable or slowly decreasing The development of weight and PAp in one of these experiments are shown in Fig 1 The difference between the experiments in group A and those in group B as regards the time taken for edema to develop, was evaluated with the Wilcoxon two sample test The 2 experiments in group A which showed a progressive increase in weight 6 h after start of perfusi... were given ranking numbers next to those which had developed edema w... period The experiment in group A, which showed no weight increase a...

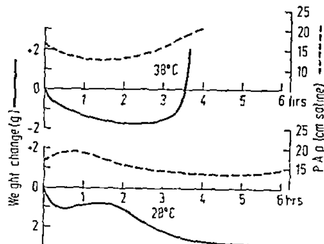


Fig 1 *Effect of perfusate temperature on development in preparation weight during perfusion in isolated and ventilated rabbit lungs* Upper diagram lung preparation perfused with plasma at 38° C, flow 280 ml/min Lower diagram lung preparation perfused with plasma at 28° C, flow 320 ml/min The pulmonary arterial pressure (PAp) level is also shown

TABLE I The effect of variations in types and temperature of perfusates on the time taken for development of spontaneous edema in isolated and ventilated rabbit lungs

Type of perfusate	Number of expts	Perfusate temperature	Time from start of perfusion to development of definite edema** in individual experiments in h
Plasma	7	38 C	$1\frac{1}{2}$, $3\frac{3}{4}$ 4, $4\frac{1}{2}$, > 6*, > 6*, > 6
Plasma	6	28 C	> 6 (in all 6 experiments)
Krebs Ringer albumin	4	38 C	$1\frac{3}{4}$ $1\frac{1}{2}$, $1\frac{3}{4}$ $1\frac{3}{4}$
Krebs Ringer albumin	3	28° C	$3\frac{1}{2}$ $4\frac{1}{4}$ > 6*

* Experiments which had not reached the edema level after 6 h of perfusion but did show an g weight at this point of perfusion termination

** Definite edema was said to be present when there had been a 4 g increase in preparation weight in the lowest weight observed during the perfusion

given the ranging number that gave the least level of significance The test then showed a significant difference between the experiments in the two groups ($p <$ 0 02)

Experiments at 38° C with 4 % albumin in Krebs Ringer (4 % AKR) solution perfusate Four lung preparations were perfused with the 4 % AKR solution at 38° C The PAp did not exceed 23 cm of water in any of the experiments The weight increase started as early as about 30 min after start of perfusion, and edema as defined in Methods had developed within 2 h in all the preparations (Table I)

Experiments at 28° C with 4 % AKR perfusate Three lung preparations were perfused with the 4 % AKR-solution at 28° C The PAp was kept below 25 cm of

TABLE II The time taken for spontaneous edema to develop in lung preparations perfused at 38° C with erythrocytes resuspended in an albumin (4 %) Krebs-Ringer-solution

Type of erythrocytes	Hematocrit in %	Perfusate volume in ml	Number of expts	Time from start of perfusion to development of definite edema* in individual experiments in h
rabbit	10—11	200	2	1¾, 2¼
horse	10—11	200	2	2¼, 2' [illegible]
rabbit	10—11	450	2	2¼, 3
horse	10—11	450	2	2¼, 2' [illegible]
horse	30—40	200	4	2¼, 3½, 4¼, 6

* Edema defined as in Table I

water A starting weight increase was observed after 1—1 1/2 h of perfusion The edema level of weight increase was in 2 of the experiments reached after 3 and 4 1/2 h of perfusion respectively In the third experiment this level was not reached within 6 h of perfusion although the weight had then been slowly increasing for 5 h

Experiments at 38° C with 4 % AKR in which erythrocytes had been resuspended In most experiments with erythrocytes in the perfusate tri-cresol or adrenaline to be added in order to counteract the spontaneous initial vasoconstriction in the pulmonary vasculature (Hauge Lunde and Waaler 1966) In 2 expts of this type papaverine was added for the same reason

In a first group of 4 expts the hematocrit of the perfusate was 10—11 %, and the volume of recirculating perfusate 200 ± 5 ml Rabbit erythrocytes were used in 2 of these experiments horse erythrocytes in the other 2 The PAp was kept below 30 cm of water In all experiments an initial decrease in weight was observed followed by a weight increase starting 1—1 1/2 h after the beginning of the perfusion In Table II is shown the time from start of perfusion until the 4 g weight increase had been reached in these experiments

In another series of 4 expts the hematocrit in the perfusate was again 10—11 % but the volume of recirculating perfusate was now 450 ± 10 ml The PAp was kept below 30 cm of water In Table II are again listed the times from start of perfusion until the defined level of edema had been reached There was no significant difference between experiments with a perfusate volume of 450 ml and experiments with a perfusate volume of 200 ml as regards the time taken for edema to develop ($p = 0.11$ There was however a statistically significant difference in the time [illegible] % AKR and [illegible] % AKR $p <$ [illegible] 001 Measured [illegible] Table I and II No difference seemed to exist between the perfusates with rabbit erythrocytes and those with h[illegible] erythrocytes as regards edema prevention (Table II)

In 4 expts horse erythrocytes were added to 4 % AKR until a hematocrit of 30—40 % was obtained The perfusate volume was kept at 200 ml The PAp [illegible]

kept below 30 cm of water The time taken for a 4 g edema to develop (Table II) differed significantly ($p < 0.01$) from those seen in the experiments with a 10—11 % hematocrit

Discussion

The method for detecting and following edema formation through the weight increase of the preparation is an indirect one Changes in the large pulmonary intravascular volume may occur under certain conditions and will then result in changes in preparation weight One important methodological problem is to discern such changes from those due to extravascular fluid accumulation The accelerating and marked weight increase observed in many of the present experiments could however, hardly have resulted from anything else than extravascular accumulation of fluid, i e edema formation Long term slow changes in the weight of a preparation are more difficult to interpret However, preparations with a weight increase of 4 g or more always showed macroscopic signs of edema when examined afterwards The nearly stable weight observed for h during perfusions with plasma at 28° C most probably reflected that no or only small net transvascular fluxes of fluid took place Some reduction in intravascular volume and a corresponding accumulation of extravascular fluid may theoretically also have resulted in a stable weight It would be strange though if these two types of changes should have been exactly equal over a period of several h Furthermore one lung preparation, similar to the present ones, has been perfusion fixed for electronmicroscopic examination after 4 h of perfusion with plasma at 28° C The ultrastructure of the lungs appeared completely normal without traces of interstitial edema (Hauge and Nicolaysen 1971)

The outflow pressure was always kept low in the present preparation at about 1—2 cm of water The inflow pressure was kept nearly constant and within the physiological range High capillary hydrostatic pressures could thus hardly have caused the edema formation observed Changes in interstitial osmotic or interstitial hydrostatic pressures did probably take place during edema development It appears less likely though that such changes should have been primary events in this connection The edema formation observed in the present experiments has therefore probably been a result of increased capillary permeability to large molecules such as proteins The present experiments and also series of previous experiments (Waaler 1971) have indicated that adrenaline papaverine or tri cresol in the doses used do not interfere with capillary permeability in these lung preparations

The course of the various types of perfusions carried out seems to indicate therefore that the normal capillary permeability to proteins has to be continuously maintained through a dynamic interplay between the endothelium and the perfusate Without a satisfactory interplay of this sort edema will develop A 4 % AKR perfusate is apparently highly deficient in this respect Plasma is a much better perfusate for the type of capillary maintenance required Also the separated addition of erythrocytes to the 4 % AKR perfus[illegible] retards edema development The significant beneficial effect of reducing the plasma perfusate temperature to 28° C suggests that

biochemical processes are involved in the maintenance of the critical endothelial characteristics

Lunde (1967) found a relationship between hematocrit in perfusates consisting of erythrocytes resuspended in plasma and the time elapsing before spontaneous edema had developed However, he did not investigate whether this effect was related to the concentration of the erythrocytes or to the total number of erythrocytes recirculating in the preparation In the present tests the hemaocrit of 4 % AKR perfusates was shown to be of importance, whereas the total number of recirculating erythrocytes (volume of perfusate) was without influence on the preparation preservation

When the temperature of the AKR was reduced from 38° C to 28° C, edema developed more slowly The start of edema development was not much delayed, but *the rate of extravascular fluid accumulation was slower at 28° C than at 38° C* It is known that edema formation due to increased capillary permeability can be rapid even at 28° C (Nicolaysen 1971) Thus the time course of the present AKR experiments indicates that the processes which lead to the increased permeability are proceeding more slowly at 28° C than at 38° C A significant effect of decreasing the temperature of an ACD (acid-citrate-dextrose) blood perfusate (homologous) from 38 to 25° C, as regards preservation of isolated dog lungs *in vitro*, has also been found by Veith *et al* (1967)

The results of the experiments performed with plasma at 38° C is at variance with those of Lunde (1967) He found that the use of plasma at 38° C always resulted in edema formation within 2 1/2 h In the present series survival times of more than 3 h were observed in 6 out of 7 experiments This discrepancy is difficult to explain Neither can the great variation in duration observed in the present plasma experiments (Table I) at 38° C be explained Since the same plasma was used in all these experiments it appears that there is some property of the preparations which is not constant

Unfortunately the present experiments did not reveal which factors in the perfusates were critical for the maintenance of normal capillary permeability in this lung preparation Studies on other organs have indicated that perfusate albumin has an important role in capillary maintenance (Landis and Pappenheimer 1963) In the present experiments the mere presence of albumin in a balanced and buffered ionic perfusate was totally unable to maintain normal capillary permeability Factors other than albumin must thus have been the more important ones here

Perfused organs or tissues are often used for studies of capillary exchange and capillary permeability Apparently great care should be taken to ensure that one is not carrying out tests in preparations where uncontrolled changes in permeability characteristics are occurring with time of perfusion

Mr A Sørlie and Miss I K Nordby have given valuable technical assistance The powdered heparin was generously supplied by Novo Industri A/S Oslo and Copenhagen This wo[illegible]as been [illegible]upported by grants to the Institute of Physiology from The Norwegian [illegible] Card[illegible]ascular Diseases

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Heart Rate in Relation to Sympathetic Efferentation Associated with the Bainbridge Reflex in Dogs

By

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An increase in the sympathetic cardiac postganglionic discharges with an associated tachycardia was detected in dogs with increased venous filling A positive correlation was found between the mean heart rate and the mean number of efferent sympathetic impulses per time unit

Bainbridge (1915) has remarked on cardiac acceleration in dogs in response to intravenous volume loads It has been suggested that this so-called Bainbridge reflex is of pulmonary parenchymal origin (Neil 1961) However by the employment of pulsatile stretching of the left atrium Ledsome and Linden (1967) have demonstrated the atrial origin of the reflex Brambring *et al* (1969) have found an almost a linear correlation between the heart rate and the mean frequency of atrial A-type impulses Hakumaki (1970) has observed a positive correlation between the number of left atrial A type impulses and the sympathetic cardiac efferent impulses The present report is aimed at demonstration and quantitation of the inter relations of the heart rate and sympathetic cardiac efferentation in dogs in which the Bainbridge reflex was elicited by i v injections of saline

6 young mongrel dogs of both sexes and weighing 5 9—10 6 kg were used The dogs were premedicated with morphine hydrochloride and anesthetized with i v chloralose followed by artificial positive pressure ventilation After thoracotomy on the left side the sympathetic postganglionic multifibre action potentials were

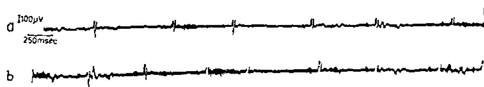


Fig 1 Heart rate and sympathetic cardiac postganglionic efferentation in a dog (6 5 kg) before (a) and 30 s after the injection of 50 ml of saline within 20 s (b) A rise in the sympathetic discharge of impulses per tim

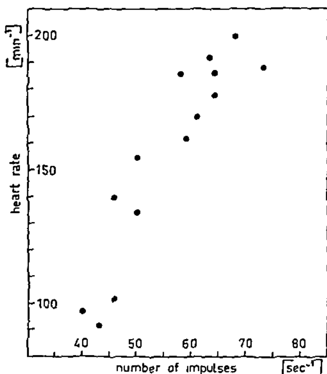


Fig 2 Mean heart rate as a function of mean number of sympathetic efferent postganglionic cardiac impulses per second in a dog (10 5 kg), during and following an i v saline injection of *120 ml* within 60 s Each point represents a sample of 2—4 s

recorded from the cranial end of the left dorsal cervical cardiac nerve, after its distal connection to the heart had been severed The mean heart rate and the mean number of sympathetic impulses per second were calculated from 2 to 4 s samples of the relevant recordings The volumes of saline injected into the left femoral vein were 50—120 ml within 20—60 s The temperature of the fluid injected was maintained at the level of the left atrial blood

In the 6 dogs studied the heart rate before injections ranged from 69—92/min This falls within the range reported as usually existent in normal unapprehensive dogs (Murphy 1942 Altman *et al* 1959) The maximum heart rate following the injections varied from 120—210/min The mean number of sympathetic efferent impulses before and following the injections ranged from 1—49/s and from 34—112/s, respectively A typical reaction pattern is illustrated in Fig 1

When the heart rate and the mean number of the sympathetic efferent impulses per second were calculated and plotted against each other, a positive correlation between these two variables was found consistently The relationship is exemplified by Fig 2

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Hyperexcretion of Catecholamines Induced by Indomethacine

By

L STJÄRNE

It was recently reported that the anti-inflammatory agent, indomethacine (Indomee®, *Merck Sharp & Dohme), blocks the release of prostaglandin* E_2 *(PGE_2)* induced by adrenaline (A), in the isolated perfused dog spleen (Ferreira, Moncada and Vane 1971) This drug might thus be a valuable tool in the study of the physiological functions of PGE_2, by allowing analysis of the picture resulting from a pharmacological block of its release *in vivo*

Recently considerable experimental evidence has been obtained at this Department, in work with isolated tissues from various species, supporting the hypothesis originally proposed by Hedqvist (1969) that the secretion of sympathetic neurotransmitter may be controlled by PGE_2 locally produced and released as a result of sympathetic nerve activity As initially shown by Wennmalm in work with the isolated perfused rabbit heart (*cf* Wennmalm 1971) blocking of the local formation of PGE_2 leads to considerable increase in the outflow of noradrenaline (NA) induced by sympathetic nerve stimulation (Samuelsson and Wennmalm 1971) Subsequently, similar observations were made in the rat vas deferens (Swedin 1971) and in the cat spleen (Hedqvist Stjarne and Wennmalm 1971)

In the present experiments indomethacine was used with the aim to block the formation of PGE [illegible] in thet rat *in vivo* The urinary excretion of NA under resting conditions as well as during intermittent exposure to cold, was used as an index of secretory activity mainly in the sympathetic nerves

12 female Sprague Dawley rats with an initial body weight of 200 g were used for the experiment Food and water were given *ad libitum* After an initial week of adjustment to the metabolic cages 6 of the rats were given daily doses of 1—15 mg/kg of indomethacine orally while the contro's received the corresponding amount of vehicle After two 24 h periods at room temperature the rats were transferred to the cold room (+2° to +4°) for 15 h each 24 h period Urine was collected daily and the excretion of free A and NA was determined fluorimetrically after purification on alumina On the 8th day 2 of the indomethacine-treated rats died The experiment was then immediately terminated

The remaining drug-treated and control rats were decapitated under brief ether anesthesia and brain heart spleen and adrenals were rapidly dissected out, weighed and homogenized in 5 volumes of 10 % trichloroacetic acid After purification on

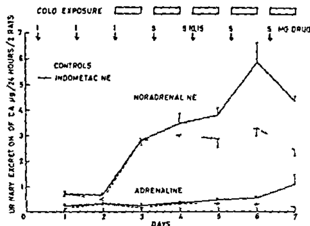


Fig 1 Urinary excretion of A and NA during indomethacine treatment, before and during intermittent exposure to cold Each point represents 6 rats 2 in each of 3 cages Mean ± SE

alumina cathecholamines were estimated fluorimetrically Recoveries were monitored by addition of tracer amounts of H^3 NA, the values given are corrected for losses (A and NA in urine represent uncorrected values)

There was no significant difference in excretion of either A or NA between controls and indomethacine treated rats at a dose level of 1 mg/kg neither at room temperature, nor during cold exposure (Fig 1) In the cold both groups increased their excretion of NA 3—4 fold while the A level was initially unchanged (*cf* Leduc 1961) As the dose was raised to 5 mg/kg there was a gradual increase in NA to a level significantly above that of the controls ($p < 0.001$) Occasional increase of the dose to 10 or 15 mg/kg did not further raise the excretion of NA During the last 2 days of the experiment the A excretion of the treated rats rose there was a moderate reduction in their urine volume they did not eat as well as previously and the volume of feces was markedly reduced Their spontaneous motor activity declined they appeared flaccid on handling and on the 8th day 2 of the rats died Within 2 h the experiment was terminated for both groups

The treated rats weighed less than the controls Peritoneal exsudate was found in the 2 rats which died spontaneously Enormous distention of the stomach was seen in all of the indomethacine treated rats Their adrenal glands were markedly enlarged when compared to those of the controls There was no difference in weight of brain or heart The NA content of the brain in the treated group was not different from that of the controls while the NA content of spleen heart and adrenals was somewhat low when compared to the control (Table I) The A content of the adrenals was not markedly changed in the indomethacine group

The present experiment shows that a sufficiently high dose of indomethacine induces urinary hyperexcretion of NA probably reflecting sympathetic hypersecretion As mentioned above the drug has been shown to block the formation and/or release of PGE in isolated tissues Furthermore inhibition of PGE formation in isolated tissues leads to increased release of NA on sympathetic nerve stimulation Thus

TABLE I Organ content of catecholamines *

	Heart NA µg/g	Spleen NA µg/2 spleens	Brain NA µg/g	Adrenals	
				NA µg/2 pairs	A µg/2 pairs
Control rats	1 31 (1 15—1 45)	1 26 (1 25—1 29)	0 66 (0 65—0 67)	9 7 (8 4—11 5)	59 4 (51 2—68 5)
Indomethacine treated rats	0 90 (0 89—0 91)	1 13 (1 10—1 16)	0 68 (0 67—0 69)	4 2 (3 9—4 5)	56 5 (44 0—67 0)

* *Means and range Originally 6 rats in each group 2 in each of 3 cages The 2 rats which died* spontaneously are not included in the Table

it is distinctly possible that the hyperexcretion of NA induced by indomethacine was due to disinhibition of the secretion of NA from sympathetic nerves, by functional suppression of PGE If this turns out to be the case the present results appear to represent the first demonstration *in vivo* that sympathetic neurotransmitter secretion, at least during sympathetic hyperactivity, is normally restricted by a PGE-mediated braking system

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